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INTERNATIONAL VALIDATION OF THE COMET ASSAY AND A HUMAN INTERVENTION STUDY

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To my mother Linda

ABSTRACT

The comet assay is an established, sensitive method extensively used in biomonitoring studies. The methods' advantages include; **a)** only a small cell sample is required, **b)** possibility to measure damage in practically any cell type, **c)** ability to measure heterogeneity in response within a cell population, **d)** relatively fast and economical procedure, and **e)** various applications of the method, which allow measurement of a range of different DNA lesions as well as DNA repair.

Several guidelines for the comet assay have been published, but no standardised protocol exists as yet. There are considerable differences between the protocols used by different research groups, which negatively affect inter-laboratory comparisons of results. Several experts in the field have highlighted the need for multi-laboratory, international validation studies, to assess intra- and inter-laboratory reproducibility and to investigate sources of variability in the results.

The papers in this thesis can be divided into two parts; one part that deals with international inter-laboratory validation studies and methodological aspects of the comet assay (paper I-III), and the other part covers a human intervention study with antioxidant capsules consisting of many different antioxidants in low doses for which the comet assay has been applied (paper IV-V).

The inter-laboratory validation trials in papers I-II indicate that the participants can detect dose-responses of both DNA breaks and oxidatively damaged DNA in coded cells, but that there is a large inter-laboratory variation in the reported values. This variation can in part be explained by differences in comet assay protocols and in image analysis. The inter-laboratory variation was decreased, but not completely removed, by calibration with ionising radiation.

In paper III we verified that several protocol steps significantly affected the outcome of the comet assay, including **a)** density of the agarose gel, **b)** extent of enzymatic incubation, **c)** duration of alkaline treatment, and **d)** time of electrophoresis, as well as the strength of the electric field applied.

In a parallel placebo-controlled, double-blind intervention study, overweight middle-aged men were supplemented for six weeks with a multivitamin supplement consisting of a range of antioxidants in doses resembling those achieved by a healthy diet (paper IV-V). In spite of elevated levels of seven out of eight measured antioxidants in the blood, the intervention did not affect the level of oxidation of lipids or DNA. Many intervention studies with good design report similar null findings. It is preferred to consume antioxidants through a healthy diet, and dietary supplements are not recommended for cancer prevention.

LIST OF PUBLICATIONS[1]

Last name changed from Johansson to Ersson in 2010.

I. Forchhammer L, Johansson C, Loft S, Möller L, Godschalk RW, Langie SA, Jones GD, Kwok RW, Collins AR, Azqueta A, Phillips DH, Sozeri O, Stepnik M, Palus J, Vogel U, Routledge MN, Handforthe C, Allione A, Matullo G, Teixeira JP, Costa S, Riso P, Porrini M, Møller P. Variation in the measurement of DNA damage by comet assay measured by the ECVAG inter-laboratory validation trial. Mutagenesis, 2010; 25(2): 113-23.

II. Johansson C, Møller P, Forchhammer L, Loft S, Godschalk RW, Langie SA, Lumeij S, Jones GD, Kwok RW, Azqueta A, Phillips DH, Sozeri O, Routledge MN, Charlton AJ, Riso P, Porrini M, Allione A, Matullo G, Palus J, Stepnik M, Collins AR, Möller L. An ECVAG trial on assessment of oxidative damage to DNA measured by the comet assay. Mutagenesis, 2010; 25(2): 125-32.

III. Ersson C, Möller L. The effects on DNA migration by altering parameters in the comet assay protocol, including agarose density, electrophoresis conditions as well as durations of enzyme and alkaline treatments. Mutagenesis, 2011; in press. Doi: 10.1093/mutage/ger034.

IV. Rytter E, Johansson C, Vessby B, Sjödin A, Möller L, Åkesson B, Basu S. Biomarkers of oxidative stress in overweight men are not influenced by a combination of antioxidants. Free Radic Res, 2010; 44(5): 522–8.[2]

V. Johansson C, Rytter E, Nygren J, Vessby B, Basu S, Möller L. Down-regulation of oxidative DNA lesions in human mononuclear cells after antioxidant supplementation correlates to increase of gamma-tocopherol. Int J Vit Nutr Res, 2008; 78(4/5): 183-94.[3]

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[1] Additional publications:

1. Ersson C, Thorman R, Rodhe Y, Möller L, Hylander B. DNA damage and oxidative stress in salivary gland tissue of chronic kidney disease patients. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2011; 112(2): 209-15.
2. Rytter E, Vessby B, Åsgård R, Ersson C, Moussavian S, Sjödin A, Abramsson-Zetterberg L, Möller L, Basu S. Supplementation with a combination of antioxidants does not affect glycaemic control, oxidative stress or inflammation in type 2 diabetes subjects. Free Radic Res, 2010; 44(12): 1445-53.
3. Rytter E, Vessby B, Åsgård R, Johansson C, Sjödin A, Abramsson-Zetterberg L, Möller L, Basu S. Glycemic status in relation to oxidative stress and inflammation in well-controlled type 2 diabetes subjects. Br J Nutr, 2009; 101(10): 1423-6.
4. Nagy E, Johansson C, Zeisig M, Möller L. Oxidative stress and DNA damage caused by the urban air pollutant 3-NBA and its isomer 2-NBA in human lung cells analyzed with three independent methods. J Chromatogr B Analyt Technol Biomed Life Sci, 2005; 827(1): 94-103.

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LIST OF ABBREVIATIONS

A	adenine
A549	human type II alveolar epithelial cell line
AlkA	3-methyladenine glycosylase II
ALS	alkali-labile sites
AP sites	apurinic and apyrimidinic sites
ATBC	α-tocopherol, β-carotene cancer prevention study
a.u.	arbitrary units
bp	base pair
BER	base excision repair
C	cytosine
CARET	the β-carotene and retinol efficacy trial
CKD	chronic kidney disease
COMICS	comet assay and cell array for fast and efficient genotoxicity testing
CV	coefficient of variation
dG	2'-deoxyguanosine
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA breaks	includes DNA strand breaks and alkali-labile sites
EC	electrochemical detection
ECNIS	Environmental Cancer Risk, Nutrition and Individual Susceptibility
ECVAG	the European Comet assay Validation Group
EndoIII	endonuclease III
ESCODD	the European Standards Committee on Oxidative DNA Damage
EtBr	ethidium bromide
FISH	fluorescent *in situ* hybridisation
Fpg	formamido pyrimidine DNA glycosylase
G	guanine
GC	gas chromatography
Gy	gray
H_2O_2	hydrogen peroxide
HD	hemodialysis
HeLa	a human transformed epithelial cell line

HPLC	high performance liquid chromatography
LMP	low melting point
MDA	malondialdehyde
MS	mass spectrometry
NER	nucleotide excision repair
NHANES	national health and nutrition examination survey
Ogg1	8-oxoguanine DNA glycosylase
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
8-iso-$PGF_{2\alpha}$	8-isoprostaglandin $F_{2\alpha}$
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI	propidium iodide
Ro	photosensitiser Ro 19-8022
ROS	reactive oxygen species
SELECT	the selenium and vitamin E cancer prevention trial
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SU.VI.MAX	the supplémentation en vitamines et minéraux antioxydants study
T	thymine
TBA	thiobarbituric acid
THP-1	human acute monocytic leukaemia cell line
UNG	uracil-DNA glycosylase
U	uracil
UV	ultraviolet (light)
YOYO-1	YOYO®-1 iodide

1 INTRODUCTION

The human genome, deoxyribonucleic acid (DNA), can be oxidatively modified by many different compounds, but reactive oxygen species (ROS) are generally regarded to be the most important cause of oxidation. An increased production of ROS has been suggested to influence the development of more than a hundred human diseases [1], and they seem to be particularly fundamental to the development of cancer and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [2]. ROS, are chemically reactive molecules containing oxygen, which also include free radicals, and are formed via many pathways in the normal metabolism of aerobic organisms. Processes involved in the production of ROS include endogenous sources such as phagocytic defence functions, leakage from mitochondrial respiration, enzymatic metabolism and the catalytic activity of transition metals [3]. In addition, ROS can be created by exogenous sources such as environmental and occupational exposure to radiation, chemicals, particles etc. Cells protect themselves against ROS by antioxidants, e.g. phytochemicals and vitamins, antioxidant enzymes, e.g. superoxide dismutase (SOD), catalase, glutathione peroxidase, repair mechanisms and adaptation (changes in expression of protective genes) [3]. In addition, DNA is also protected through its supercoiled structure and histones.

Oxidative stress has been defined as a disturbance in the pro-oxidant-antioxidant balance in favour of the former [4], leading to potential damage; i.e. the level of oxidising agents (primarily ROS) overwhelm the protective antioxidant defence systems and repair capacity of the cell. ROS are short-lived and therefore complicated to measure, especially *in vivo*. However, analysis of sufficiently stable end-products from oxidation processes, such as oxidatively damaged lipids, proteins and DNA, can give an estimate of the level of oxidative stress in cells [5].

Environmental and occupational genotoxins can damage DNA in many different ways creating a range of DNA lesions such as oxidation damage, alkylation damage, base loss, DNA cross-links, covalent bonding of bulky DNA adducts etc. Many different types of DNA lesions are used as indicators of biological states or processes, subsequently referred to as biomarkers, and provide valuable information about exposure to damaging or protective agents, individual susceptibility, early stages of diseases etc. The most frequently measured biomarker for oxidatively damaged DNA is 8-oxoguanine, which was discovered in 1984 by Kasai and Nishimura [6]. The great interest in this biomarker is attributed to that it is believed to be important in carcinogenesis [7]. 8-oxoguanine, one of the most abundant DNA lesions, causes mutations by inducing guanine-cytosine (G-C) to thymine-adenine (T-A) transversions.

8-oxoguanine has been studied extensively and there is a range of different methods available to measure this lesion.

The comet assay is a method with which several biomarkers of exposure (reflecting the biologically effective dose) can be measured [8]. The alkaline comet assay is an established, sensitive method extensively used in genetic toxicology, which in its basic form measures DNA breaks, i.e. strand breaks and alkali-labile sites (ALS), but can also be modified to measure a wider range of different lesions. Specific types of DNA lesions can be measured by using lesion-specific repair enzymes [9], such as formamido pyrimidine DNA glycosylase (Fpg) [10], endonuclease III (EndoIII) [11], human 8-oxoguanine DNA glycosylase (hOgg1) [12], 3-methyladenine glycosylase II (AlkA) [13], T4 endonuclease V [14, 15] and uracil-DNA glycosylase (UNG) [16], which enables measurement of oxidatively damaged bases, alkylated bases, cyclobutane pyrimidine dimers, and misincorporated uracil. Recently, the comet assay has been modified to measure the DNA repair capacity of cells; i.e. base excision repair (BER) of mainly 8-oxoguanine induced by the photosensitiser Ro 19-8022 (Ro) and visible light [17] or nucleotide excision repair (NER) of bulky DNA adducts [18], DNA damage induced by ultraviolet (UV) C light [19], and DNA cross-links [20]. The kinetics of repair of DNA damage can also be estimated by exposure to different agents *ex vivo*, e.g. hydrogen peroxide (H_2O_2), and monitoring the rejoining of strand breaks or repair of DNA lesions. Other modifications of the comet assay allow the detection of DNA-DNA cross-links [21], and gene-specific DNA damage in combination with the fluorescent *in situ* hybridisation (FISH) methodology [22]. The many applications of the comet assay together with the **a)** low number of cells required, **b)** ability to measure heterogeneity in sensitivity or response between cells, **c)** sensitivity in detecting low levels of DNA damage, and, **d)** possibility to measure damage in practically any eukaryotic cell type are some of the reasons for the assay's increasing popularity [23]. Figure 1 shows the amount of published comet papers each year over the last 20 years. Today, the comet assay is one of the most frequently used methods to measure DNA damage in environmental toxicology.

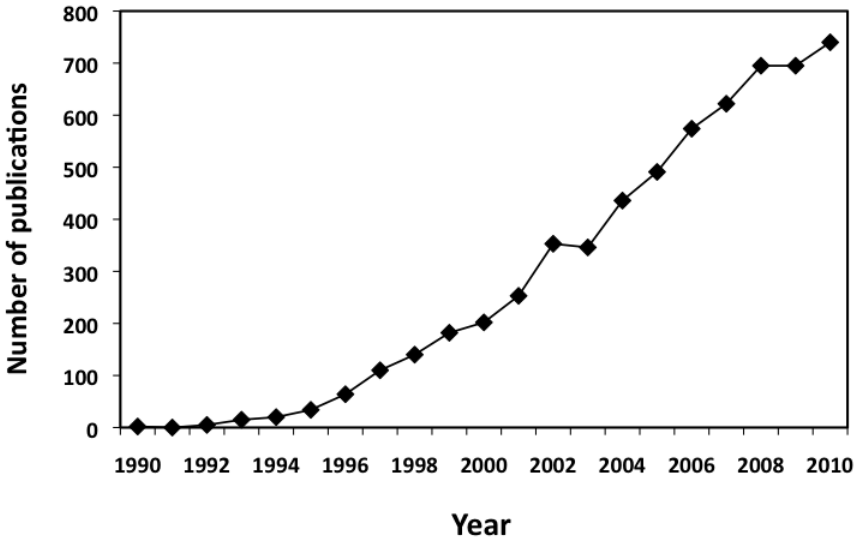

Figure 1. The amount of scientific articles published each year during the last 20 years (based on a PubMed search of the "comet assay" 2011-08-10).

The comet assay is widely used in *in vitro* studies using cultured cells and in *in vivo* studies where DNA lesions can be measured in any eukaryotic cell type where a single cell suspension can be obtained. Since a relatively small amount of cells are required for analysis, the method is particularly suitable for *in vivo* studies. One important field where comet assay can be applied is in intervention studies assessing the impact of nutrition. In humans, the assay is mainly applied on peripheral blood mononuclear cells (PBMC). The comet assay has been shown to be a highly sensitive method to identify carcinogens and non-carcinogens [24, 25]. Although the range of detection is rather narrow for the assay, approximately a hundred to several thousand breaks, it conveniently covers physiologically relevant levels of damage, ranging from the normal background levels of damage and damage levels induced by non-lethal doses of damaging agents [26].

Several guidelines for the comet assay have been published [27-31]. However, no standardised protocol exists as yet and there are considerable differences in the protocols used by different research groups, which negatively affect inter-laboratory comparisons of results. Although the inter-laboratory variability is well-known, few studies have addressed this issue. Several experts on the field have highlighted the need for multi-laboratory international validation studies to assess intra- and inter-laboratory reproducibility and to investigate sources of variability of results [8, 29, 31].

The papers in this thesis can be divided into two parts; one part that deals with international inter-laboratory validation studies and methodological aspects of the comet assay (paper I-III) and the other part covering a human intervention study with antioxidant capsules consisting of many different antioxidants in low doses for which the comet assay has been applied (paper IV-V).

2 RESEARCH AIMS

The general aims of this thesis were to present and investigate the possibilities and limitation of the comet assay, and to investigate whether the level of oxidative stress could be decreased by supplementation of a mixture consisting of a range of antioxidants in low doses in a randomised parallel double-blind placebo-controlled intervention study.

Specific aims:

a) To investigate inter-laboratory variations in DNA breaks and Fpg-sensitive sites measured by the comet assay (papers I and II).
b) To assess the possibility of reducing the variation between laboratories through calibration with reference samples (papers I and II).
c) To assess the variability in scoring and staining by analysis of slides prepared by one laboratory (paper I).
d) To investigate the impact on DNA migration by different comet assay protocols (papers II and III).
e) To assess the possibility of reducing the variation between different protocols through calibration with reference samples (paper III).
f) To examine the possibility of modifying the level of oxidative stress in healthy, middle-aged, over-weight males by supplementation with a mixture of antioxidants in low doses (paper IV).
g) To examine the ability of α- and γ-tocopherol to protect against oxidative DNA lesions (paper V).

3 THE ANALYTICAL METHOD COMET ASSAY

The focus throughout this thesis is on the alkaline (pH>13) comet assay and Fpg comet assay since these applications have been used in the constituent papers. The alkaline (pH>13) comet assay, and utilisation of enzymes for assessment of specific lesions, are described in section 3.2. In addition, a number of other applications of the comet assay will be briefly described, including assessment of **a)** DNA damage in specific genes in combination with the FISH methodology (section 3.3), **b)** DNA-DNA cross-links (section 3.4), and **c)** DNA incision activity reflecting BER and NER (section 3.5). From now and onwards, the alkaline comet assay (pH>13) will be referred to as the alkaline comet assay, whereas the alkaline comet assay combined with enzyme treatment are referred to as the Fpg comet assay, the EndoIII comet assay etc, depending on the enzyme.

3.1 A BACKGROUND TO THE DIFFERENT VERSIONS OF THE COMET ASSAY

The principles of the comet assay was first introduced in 1984 by Östling & Johanson [32]. This original protocol is often referred to as neutral although a pH of 9.5 was employed. Although this value is far above what is normally referred to as neutral pH (pH 7), it is nonetheless insufficient to unwind DNA. A few years later, Singh *et al.* described the alkaline comet assay, including lysis in a buffer with high salt concentration and a subsequent 20 min of alkaline treatment in a strong alkaline solution (pH>13), followed by 20 min of electrophoresis in the same solution [33]. In 1989, Olive *et al.* introduced a version of the comet assay where slides were submerged in a neutral solution consisting of Tris/EDTA buffer before electrophoresis [34]. In 1990, Olive *et al.* introduced yet another version of the comet assay using a weak alkali solution (pH~12.3) [35]. Today, the alkaline (pH>13) version of the assay, which detects DNA strand breaks and ALS, is most frequently used and is considered the optimal version for identification of genotoxic agents [31]. Several guidelines for the comet assay have been published [27-31], but there is no standardised protocol and there are considerable differences in protocols used by various research groups. Many, but not all, protocols are covered by the general procedure described in the following section. The specific protocol that is currently used by the author of this thesis is described in detail in paper III.

3.2 THE GENERAL PROCEDURE OF THE ALKALINE AND ENZYME COMET ASSAYS

In short, cells are embedded in agarose and lysed at pH 10, leaving a structure called a nucleoid. To specifically measure oxidatively damaged DNA (or some other types of DNA lesions described in section 3.2.4), an additional step of digestion with DNA glycosylase can be added. The glycosylase recognises and removes lesions leaving an abasic site, which is converted to a strand break either by the enzyme's attached lyase activity or by the subsequent alkaline treatment. To obtain single stranded DNA, treatment with an alkali solution is performed. Following this, electrophoresis is done under the same alkaline conditions as the previous step, where the strand breaks through their ability to relax DNA supercoiling, allow the negatively charged DNA loops to extend towards the positively charged anode. The DNA is stained and a tail (comet) of mainly single stranded DNA can be seen with a fluorescence microscope (figure 2).

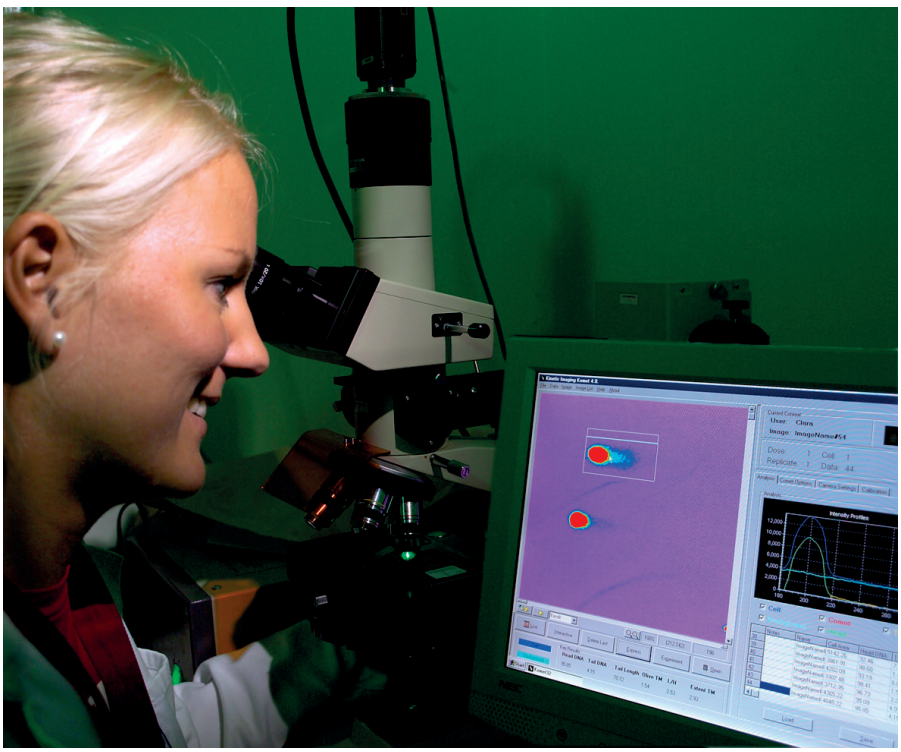

Figure 2. Comets scored with a fluorescence microscope. Photo by Staffan Larsson.

The comets are analysed either by using visual scoring or computerised image analysis. The magnitude of the DNA-tail provides information about the level of DNA lesions. A schematic description of the assay is depicted in figure 3. In the following sections, the general procedure of the comet assay is described step by step.

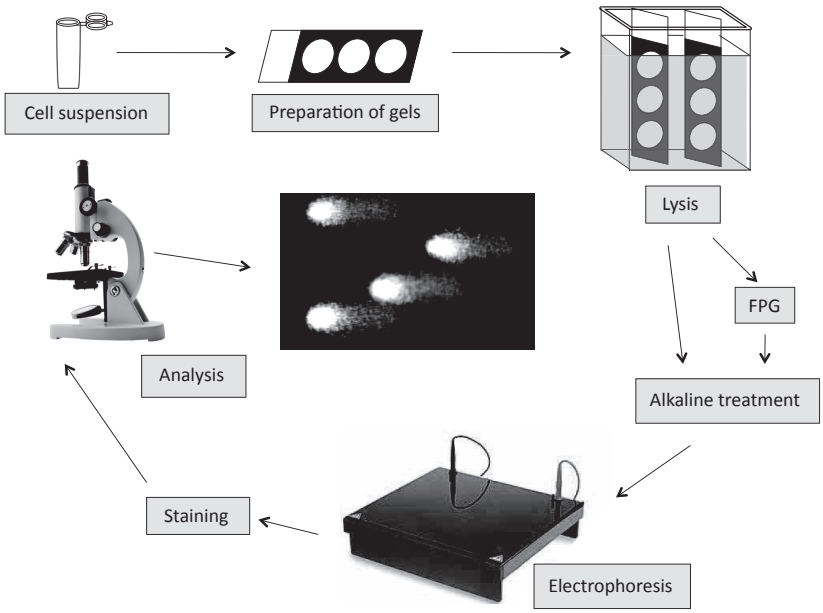

Figure 3. Schematic description of the different steps in the comet assay.

3.2.1 Cell suspension

The comet assay can be applied to basically any cells with nuclear DNA where a single cell suspension can be obtained. Cells from tissue biopsies that can be homogenised, buccal cells obtained by scraping of the buccal cavity, cells isolated from blood, whole blood, cultured cells, and even plant cells can be used, although some cell types, such as plant cells and sperm cells, require modified protocols. It is more frequent to isolate PBMC than to perform comet assay on whole blood. The PBMC can be analysed directly after isolation or be frozen down under appropriate conditions, which allows multiple analyses or simultaneous analysis of different samples, such as those collected before and after exposure. PBMC are isolated from blood by density gradient centrifugation using commercially available tubes/solutions like Lymphoprep™, Histopaque™-1077 or BD Vacutainer® CPT™. Whole blood, on the other hand, does not require centrifugation. It has recently been suggested that whole blood, frozen in small aliquots, as opposed to larger aliquots, can be used in the comet assay [36]. If this

holds true following rigorous evaluation, it would not only save time for those who are working with the comet assay, but also allow for comet assay analysis of samples archived in biobanks [36].

3.2.2 Agarose

Cells in suspension are mixed with low melting point (LMP) agarose and the mixture is allowed to solidify to a gel on a microscope slide, already pre-coated with agarose, or on a GelBond® film. When it comes to glass slides, clear ones are recommended as opposed to the fully frosted slides frequently used in the past, as the latter suffers from a high background noise [28]. Normally, one electrophoresis slide contains 1-3 agarose gels, each gel containing a few thousand cells. Recently, however, Shaposhnikov *et al.* showed how using 12 agarose mini-gels per slide substantially increased the amount of samples that can be analysed in a single experiment [37].

3.2.3 Lysis

The slides (or GelBond® films) are placed in a lysis solution for a minimum of 1 h at 4 °C. During lysis, membranes, cytoplasmic and nuclear constituents and most histones are removed. The structure remaining after lysis is called a nucleoid and consists of supercoiled DNA attached to a nuclear matrix. The lysis buffer frequently consist of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10 and 1% Triton X-100, but some researchers also add dimethyl sulfoxide (DMSO) and/or N-lauroylsarcosine. Triton X-100 is generally sufficient for cell lysis, although a second detergent might be necessary for complete lysis for some cell types [31]. Triton X-100, which act as a detergent, is added at the day of the experiment by gentle stirring, preferably for at least 1 h based on observations by the author of this thesis (but at a minimum for 15 min [38]). Hartmann *et al.* stated in 2003 that N-lauroylsarcosine was considered redundant [29].

3.2.4 Enzyme treatment

DNA glycosylases can be added to the alkaline comet assay to measure specific DNA lesions. DNA glycosylases that have been used in the comet assay include Fpg [10], EndoIII [11], hOgg1 [12], AlkA [13], T4 endonuclease V [14, 15] and UNG [16]. DNA glycosylases travel (slide) along the DNA chain and when it faces a damaged base, a structural change take place where the damaged base is partitioned forward to the active site of the enzyme where the glycosidic bond between the damaged base and the sugar is cleaved [39]. The resulting abasic site leads to a strand break either by the enzyme's associated lyase activity or by the subsequent alkaline treatment. Glycosylases are either monofunctional, i.e. they remove the damaged base by cutting the glycosidic bond between the base and the sugar, or bifunctional, i.e., in addition to removing the

damaged base by cutting the glycosidic bond, it also cut the DNA backbone [40]. Incubation of slides with only buffer or buffer containing enzyme enables an estimation of both DNA breaks and the calculation of net enzyme-sensitive sites. The enzyme most frequently used in the comet assay, Fpg, recognises and removes mainly 8-oxoguanine but also a range of other lesions. The substrates of the glycosylases that have been used in the comet assay are listed below (incomplete list). However, it should be emphasised that establishing substrates *in vitro* does not necessarily mean that the enzymes recognise all of these substrates *in vivo*. Some of the most common products of DNA base oxidation are shown in figure 4.

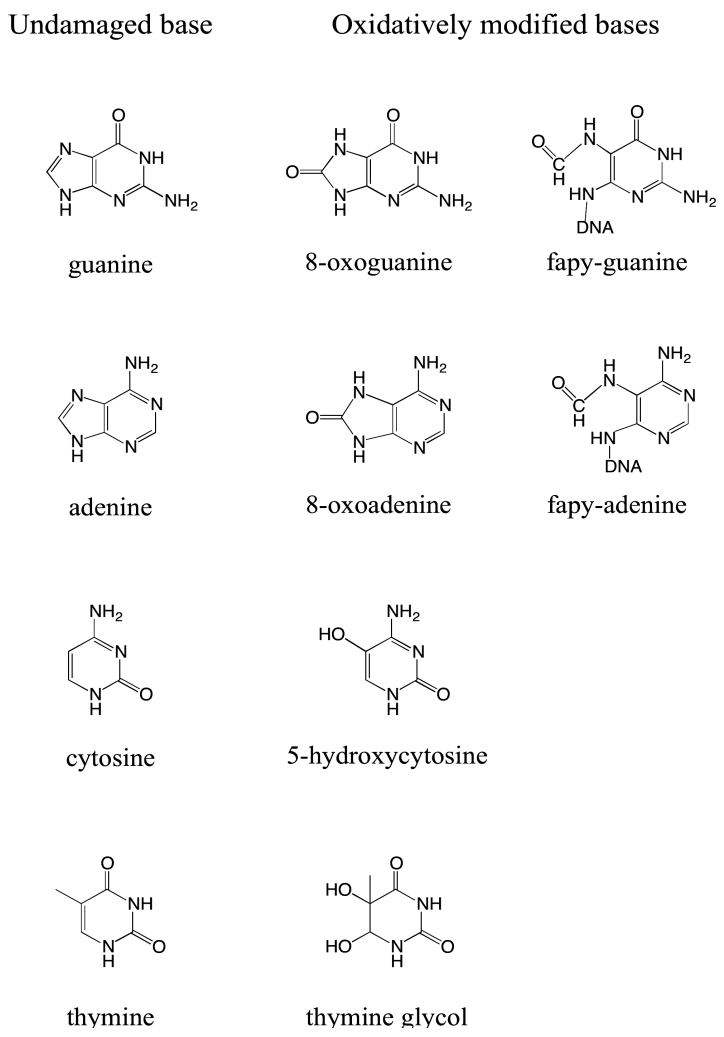

Figure 4. The DNA bases and some of the most common products of base oxidation.

Fpg

Fpg recognises and removes primarily oxidised purines such as 8-oxoguanine (which is the main substrate) and the formamidopyrimidines 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapy-guanine) and 4,6-diamino-5-formamidopyrimidine (fapy-adenine) (figure 4) [41, 42]. Fpg has also been reported to recognise a range of other lesions, e.g. 5-hydroxycytosine, thymine glycol, abasic sites, 8-oxoadenine, various kinds of ring opened 7'-nitrogen and 8'-carbon guanine adducts and alkylation damage (probably alkylation of the 7'-nitrogen of guanine) [41-44]. 8-oxoguanine can also be further oxidised into spiroiminodihydantoin and 5-guanidinohydantoin, which have also been shown to be recognised by Fpg [45]. In addition to the glycosylase activity, Fpg has associated lyase and endonuclease activities (see section 6.4.1) [42, 43, 46].

EndoIII

EndoIII recognises pyrimidine oxidation products and its main substrate is thymine glycol (figure 4). It also recognises a wide variety of other substrates including abasic sites, urea, uracil glycol, 5,6-dihydrothymine, 5-hydroxy-6-hydrothymine, 5-hydroxyuracil, 5,6-dihydrouracil, 5-hydroxy-6-hydrouracil and 5-hydroxycytosine [43, 47]. EndoIII has an associated lyase activity [43, 47].

hOgg1

hOgg1's main substrate is 8-oxoguanine, which it is considered to detect with a higher specificity than Fpg [12], although it also recognises other lesions, including abasic sites opposite cytosine, methyl-fapy-guanine paired with cytosine and 7-methyl-8-oxoguanine [46, 48]. hOgg1 has an associated lyase activity [49], although it appears to be less efficient than that of Fpg [43].

AlkA

AlkA's main substrate is 3-methyladenine but it is a very promiscuous non-specific enzyme [43, 50]. AlkA recognises a range of alkylated bases including 3'-nitrogen and 7'-nitrogen adducts of adenine and guanine, respectively, and alkylation damage of cytosin's exocyclic oxygen [43, 50]. AlkA has even been shown to attack undamaged DNA at high concentrations [50]. AlkA has no associated lyase activity [43].

T4 endonuclease V

Endonuclease V from T4 phage is a glycosylase/lyase, which has a narrow substrate specificity for UV-induced cyclobutane pyrimidine dimers, of which the *cis-syn* T-T dimer is the preferred substrate [43].

UNG

Uracil in DNA, originating from deamination of cytosine or from misincorporation into DNA [51], is recognised and excised by UNG. The glycosylase removes uracil in both double-stranded and single-stranded DNA with high specificity but has also been reported to recognise other lesions, such as 5-fluorouracil, 5-hydroxyuracil, isodialuric acid and alloxan [43, 47, 51]. UNG has no associated lyase activity [43, 47].

Other enzymes

A study assessing whether the comet assay can be modified to assess epigenetic changes using enzymes is ongoing (Nagy *et al.,* in preparation). Attempts have been made to use the enzyme complex uvrABC in the comet assay, but the enzyme failed to find sufficient levels of its substrate [10, 52].

3.2.5 Alkaline treatment

In order to unwind and denature DNA, and hydrolyse ALS, the slides are placed in an alkaline solution (0.3 M NaOH, 1 mM EDTA, pH > 13) for typically 20-40 min at 4°C. This is performed directly after lysis in the alkaline comet assay, or after enzyme treatment in the enzyme version of the comet assay.

3.2.6 Electrophoresis

Following alkaline treatment, electrophoresis is performed under alkaline conditions where the strand breaks by their ability to relax DNA supercoiling of DNA loops allow DNA to extend out from the nucleoid under the influence of an electric field [53]. Electrophoresis is typically performed for 20-30 min, often at 0.8-1.2 V/cm. The negatively charged DNA migrates towards the positively charged anode, creating an image that resembles a comet. In the lab of the author of this thesis, a black COMET-20 tank (Scie-Plas Ltd, Cambridge, UK) is used, which has ice-cold water circulating under the platform to keep the buffer in the tank at a constant temperature. It also aids in blocking light from reaching the gels as better results are obtained when this step is performed in darkness.

3.2.7 Neutralisation

DNA is neutralised, for example by placing the slides in phosphate buffered saline (PBS) or 0.4 M Tris, pH 7.4.

3.2.8 Fixation

Slides are often dehydrated by treatment with methanol or ethanol, which allows for long-term storage.

3.2.9 Staining

The DNA is stained by a DNA binding fluorescing dye, e.g. ethidium bromide (EtBr), propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), SYBR® Green, SYBR® Gold, YOYO®-1 iodide ´(YOYO-1), or by a non-fluorescing dye, i.e. silver nitrate.

3.2.10 Scoring of comets

A comet with a head of mainly supercoiled DNA and a tail of extended DNA loops and DNA fragments can be seen with a fluorescence microscope [53]. The comets are normally analysed with UV-fluorescence microscopy. The comets can be analysed by visual scoring, by computerised image analysis or by the recently developed automatic computerised analysis (discussed in section 5.1.6) [54]. There is a range of different software available to analyse comets, for example Comet Assay IV™ (Perceptive Instruments, Suffolk, UK) and Komet (Kinetic Imaging Ltd, Bromborough, UK).

3.2.11 Different endpoints

Visual scoring is reported as arbitrary units (a.u.), often in a scale 0-400. The comets are divided into five classes ranging from undamaged to very damaged cells as described by Collins *et al.* [11]. Computerised scoring is reported using a range of different endpoints. The most frequently used endpoints for computerised scoring are probably %DNA in tail, tail moment and tail length. In addition, different distributions of comet images are sometimes described. These units are discussed in chapter 5. Calibration with ionising radiation, i.e. γ-rays or X-rays, allows for primary comet assay endpoints to be reported in 'real' damage levels such as lesions/10^6 base pair (bp), which is described and discussed in sections 5.3.1 and 5.3.2.

3.3 FISH COMET ASSAY

The FISH application of the comet assay is technically demanding, but can provide unique information about the DNA damage and repair in specific genes and DNA sequences [22]. By hybridising fluorescently labelled probes to DNA after electrophoresis, DNA damage and repair in particular genes and DNA sequences can be measured by the comet assay [22, 55, 56]. If the DNA of the gene is found in the comet tail, this indicates that a DNA break has occurred in the proximity of the gene [55].

3.4 DETECTION OF CROSS-LINKS

A modified version of the comet assay can be used to measure DNA cross-links [21, 57]. This type of lesion can be highly toxic to the cell, as it can result in inhibition of DNA strand separation during replication and transcription [21]. In this assay, a known amount of strand breaks are induced in cells by radiation. When performing the comet assay, the cross-links' potential to inhibit DNA migration results in a decreased DNA migration proportional to the level of inter-strand cross-links [21].

DNA cross-links reduce the migrating capability of DNA, which can result in misinterpretation of results obtained by the regular alkaline comet assay. If suspecting that cells contain DNA-protein cross-links, an additional step can be added where the lysed cells are exposed to proteinase K before alkaline treatment in the alkaline comet assay [31].

3.5 THE *IN VITRO* COMET REPAIR ASSAY

In short, to measure DNA incision as an estimate of DNA repair capacity, a cell extract containing DNA repair enzymes is obtained from cells (frequently lymphocytes), by lysis of the membranes and addition of a buffer. Cells with induced DNA damage are embedded in agarose on a microscope slide, and lysed to expose the DNA, followed by incubation with the cell free lymphocyte extract containing DNA repair enzymes. The extract's incision activity (the first step in the repair process) on DNA damage is measured by the comet assay. Depending on the character of the induced DNA damage, BER and NER can be measured. To measure BER by Ogg1, 8-oxoguanine is induced by exposing cells to visible light when mixed with Ro [17]. To measure NER of bulky DNA adducts, damage is induced by benzo[a]pyrene-diol-epoxide [18]. To measure NER of UV-induced damage (mainly cyclobutane pyrimidine dimers and pyrimidine 6-4 photoproducts), damage is induced by UVC [19]. To measure NER repair of DNA cross-links, damage can be induced by oxaliplatin [20].

4 VALIDATION OF THE COMET ASSAY

To be able to use biomarkers as tools in cancer risk assessment, the biomarkers must be extensively validated. Validation of the comet assay is crucial for its value as a tool to identify and monitor **a)** environmental and occupational hazards, **b)** diseases linked with oxidative stress, **c)** the effects of nutrition, **d)** the effect of medical treatments, and **e)** to assess individual susceptibility [58]. The validation process is continuous and important steps include the evaluation of exposure-effect relationships in laboratory studies and in biomonitoring studies on humans, and demonstrating associations between the biomarkers and disease [59]. An important step of biomarker validation is inter-laboratory validation studies. Several experts in the field have emphasised that in order to demonstrate the comet assay's inter- and intra-laboratory reproducibility, reliability, and to investigate sources of variability in results, the method needs to undergo multi-laboratory, international validation studies [8, 29, 31]. The European Standards Committee on Oxidative DNA damage (ESCODD) and European Comet assay Validation Group (ECVAG) networks have assessed several aspects of the validation of the comet assay. Prof. Lennart Möller's research group has been a member of both ESCODD and ECVAG, established in 1997 and 2006, respectively. The first two studies performed by the ECVAG network are included in this thesis (papers I and II). The author of this thesis is involved in four additional (ongoing) ECVAG studies. The objectives of all the ECVAG studies are described in section 4.2. The findings of these studies are discussed in this and the following chapter. Some of the most important aspects assessed by the ECVAG network are **a)** precision (variation between laboratories), **b)** reproducibility (repeated analysis of identical samples), and **c)** sensitivity (the ability to measure dose-responses).

4.1 THE WORK OF ESCODD

ESCODD was established in 1997 after scientists had observed that different methods used to analyse the level of oxidative DNA lesions, i.e. 8-oxoguanine, gave different results [60]. Scientists using gas chromatography-mass spectrometry (GC-MS) techniques reported values that were about 10 times higher than those obtained using high performance liquid chromatography with electrochemical detection (HPLC-EC). Even lower levels of 8-oxoguanine were seen with enzymatic methods, e.g. the comet assay [60]. Collaborations through ESCODD were set up in order to examine differences between the methods, validate them, develop trustworthy techniques, and to reach a consensus about the true background level of 8-oxoguanine. ESCODD consisted of 27, mainly European, laboratories, which performed several inter-laboratory validation studies. Samples of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), oligonucleotides

containing 8-oxodG, calf thymus DNA and pig liver [61], 8-oxodG, untreated calf thymus DNA and calf thymus DNA with experimentally induced 8-oxoguanine [62], oligonucleotides containing different amounts of 8-oxodG but without undamaged guanine [63], 8-oxodG, 2'-deoxyguanosine (dG), untreated calf thymus DNA and calf thymus DNA with experimentally induced 8-oxoguanine [64], pig liver and untreated human transformed epithelial (HeLa) cells [65], undamaged HeLa cells and HeLa with experimentally induced 8-oxoguanine [66], and, lymphocytes from healthy men and untreated HeLa cells [67] have been analysed using different methods by the members of ESCODD. As the comet assay requires intact cells for analysis, this methodology was introduced only at a later stage into the ESCODD validation project [65-67], since the first studies were performed on DNA constituents. ESCODD reported that the enzymatic methods were less susceptible to artefact oxidation, and hence, reported variations in background levels were lower compared to HPLC-EC methods, although, the latter method was better at detecting a dose-response [66]. However, both the background levels and the dose-range were much lower for the comet assay (HeLa cells were exposed to 0-0.4 μM Ro for 2 min for the comet assay and 0-5 μM Ro for 5 min for the chromatographic methods), which precludes a direct comparison [66]. Seven out of eight laboratories reported very similar dose-response slopes when analysing HeLa cells containing 8-oxoguanine in their DNA with HPLC-EC methods, although the background levels (the intercept) varied substantially [66]. In comparison, only three out of eight laboratories could detect the dose-response relationship in net Fpg-sensitive sites using the comet assay [66]. Gedik *et al.* had previously showed that the slopes of the dose-response curves obtained by the HPLC-EC method and the comet assay, when analysing cells containing 8-oxoguanine in their DNA, were similar [68]. However, as in the ESCODD study, the background level, as well as the dose range, was substantially lower for the comet assay than for the HPLC-EC method [68]. The ESCODD network estimated the normal background level of 8-oxoguanine in human lymphocytes to be between 0.3 and 4.2 8-oxoguanines per million undamaged guanine bases [67].

4.2 THE WORK OF ECVAG

ECVAG was created within the Environmental Cancer Risk, Nutrition and Individual Susceptibility (ECNIS) network of excellence in 2006, and currently consists of 14 laboratories that perform validation studies of the comet assay. Each of these studies will result in a scientific paper. The author of this thesis is a co-author of six of these papers (studies 1-3 and 5-7 below), of which two have been published and are included in this thesis (papers I and II). Of the remaining ongoing studies, two studies have recently terminated and the manuscripts are now under preparation (studies 3 and 5). For studies 6 and 7, we have not yet received results from all participating laboratories

and all obtained results are still coded. In this section, the objectives of the seven ECVAG studies are described briefly.

Study 1 (paper I in this thesis) comprised of inter-laboratory investigations of three key areas; **a)** variation in scoring of pre-made slides to assess the influence of scoring and staining, **b)** inter-laboratory variation in analysing DNA breaks in coded samples to assess the inter-laboratory variation, and **c)** the possibility to reduce the inter-laboratory variation by using protocol-specific calibration curves [69]. The first part of this study included scoring of pre-made slides containing nucleoids with varying degrees of DNA migration. In the second part of this study, frozen human acute monocytic leukaemia THP-1 cells containing DNA breaks (for calibration curve samples and coded samples, respectively) were distributed to 12 participating laboratories that were to analyse these samples using their own comet assay protocols.

Study 2 (paper II in this thesis) aimed to investigate **a)** the participating laboratories' abilities to detect a dose-response in coded samples containing 8-oxoguanine, **b)** the inter-laboratory variation in reported Fpg-sensitive sites in coded samples, and **c)** the possibility to reduce the inter-laboratory variation by using protocol-specific calibration curves [70]. Reference cells containing DNA breaks, and coded cells containing oxidatively damaged DNA, were distributed to 10 participating laboratories, that were instructed to analyse these samples using their own comet assay protocols.

Study 3 involved eight laboratories' abilities to detect DNA repair capacity as incisions per 10^6 bp in three different cell lines using the *in vitro* comet repair assay (Godschalk *et al*., in preparation).

Study 4. The ability to reduce intra-experiment and inter-experiment variability in DNA migration by using an authentic internal standard was assessed by Zainol *et al.* [71]. In this study, Zainol *et al.* introduced a true internal standard for the comet assay, by embedding distinguishable 'reference' cells in the same gel as the test comets that were to be investigated (see section 5.3.3).

Study 5. In this study, the ability to decrease the inter-laboratory variation by adopting a set of reference conditions was assessed (Forchhammer *et al*., in preparation). In the reference protocol, the protocol parameters that were considered to be the most important were fixed, i.e. the **a)** agarose concentration (1% final concentration), **b)** duration of enzymatic incubation (30 min), **c)** time of alkaline treatment (20 min), **d)** strength of the electric field (1.2 V/cm), and **e)** duration of electrophoresis (20 min). The reference conditions also included the composition of the solutions/buffers used, as well as the dilution of the enzyme. Moreover, the participants were instructed to change

their image analysis system settings if they deviated from distributed pictures. The participating 14 laboratories analysed coded reference samples and PBMC, both with their own protocols and the reference protocol.

Study 6 is still ongoing. In this study, the intra- and inter-laboratory variation in detected DNA damage in PBMC will be assessed. By analysing three identical coded samples of PBMC from three different donors and standard curve samples on three different days, using the participants' own protocols, the intra-day, the day-to-day, and the inter-laboratory variation in DNA breaks and Fpg-sensitive sites can be assessed.

Study 7. In this ongoing study, the inter-laboratory variation in detecting DNA damage in PBMC will be assessed. The participating laboratories have received coded samples containing PBMC collected in five different countries using the same technique, and have been instructed to analyse these with their own protocols.

4.3 DOSE-RESPONSE RELATIONSHIPS IN THE ECVAG STUDIES

In general, the participants in the ECVAG studies (papers I and II) could detect dose-responses in both reference cells and coded samples. However, there was a large inter-laboratory variation in the reported primary comet assay endpoints, i.e. in the reported a.u. and %DNA in tail. This variation was attributed to both differences in comet assay protocols and image analysis. The dose-response relationships in papers I and II are presented shortly in this section, whereas influences of protocol and image analysis are mainly discussed in the next chapter.

4.3.1 Analysis of calibration curve samples

In both paper I and paper II, calibration curve samples irradiated with 0, 2.5, 5 and 10 Gray (Gy) of γ-radiation were distributed to the participating laboratories to be analysed with the participants' own protocols. In paper I, the dose-dependent relationships for the calibration curves (R^2_{median} = 0.987, range: 0.895-0.992) were significant for 11 laboratories ($P < 0.05$) and borderline significant ($P = 0.054$) for one laboratory (ANOVA). In paper II, all 10 participating laboratories reported significant dose-dependent relationships in the calibration curves (R^2_{median} = 0.970, range: 0.931-0.995, $P < 0.05$, ANOVA). Calibration curves are discussed in section 5.3.1.

In paper I, an assessment of the contribution of the intra- and inter-laboratory variation to the overall variation was made by comparing the standard curves from the three first ECVAG studies (papers I and II, and ECVAG study 3 [Godschalk *et al*., in preparation]) [69]. The contribution to the overall variation was to a large extent from

radiation (66%) followed by inter-laboratory variation (21.6%; $P < 0.001$ for both variables) [69]. In addition, the unexplained contribution of 12.3% to the overall variation, was much larger than the intra-laboratory variation, which was only 0.1% [69]. However, the study design of paper I does not allow discrimination between day-to-day and intra-laboratory variation. These two variables will, however, be assessed in ECVAG study 6 by using a Latin square design (analysis of three donors and three identical samples per donor on three different days).

4.3.2 Analysis of coded pre-made slides and cryo-preserved coded cells containing DNA breaks

In paper I, all participating laboratories detected the same ranking in the coded pre-made slides, which consisted of gel-bonds containing embedded cells exposed to 0, 2.5, 5 and 10 Gy of γ-radiation and subject to a fixed comet assay protocol at the University of Copenhagen (figure 5). Figure 5 in this thesis deviate slightly from figure 1b in paper I, since all laboratories ($n = 12$) have been included in the figure in this thesis. The largest standard deviation was seen for the highest dose of γ-radiation, with DNA migration ranging from 29.0 to 94.6 %DNA in tail or a.u. in the scale 0-100. The inter-laboratory variation in the pre-made slides was surprisingly high, which indicated that the image analysis, perhaps together with staining of cells, is an important parameter affecting the outcome of the comet assay. Since the pre-made slides consisted of the same cells as the coded cryo-preserved samples, an attempt to assess the contribution of the image analysis could be made by a statistical model (where the level of DNA damage in the coded samples was the dependent variable, the laboratory categorical variable, and the dose of γ-radiation and the DNA damage in the pre-made slides continuous variables). All factors significantly predicted the level of DNA migration: dose of γ-radiation ($P < 0.001$), laboratory ($P < 0.001$), and the reported levels of DNA migration in the pre-made slides ($P < 0.01$), which indicated that even though the dose of γ-radiation was the strongest predictor, both image analysis and comet assay protocol significantly affected the outcome of the comet assay [69]. The impact of protocols and image analysis is further discussed in the following chapter.

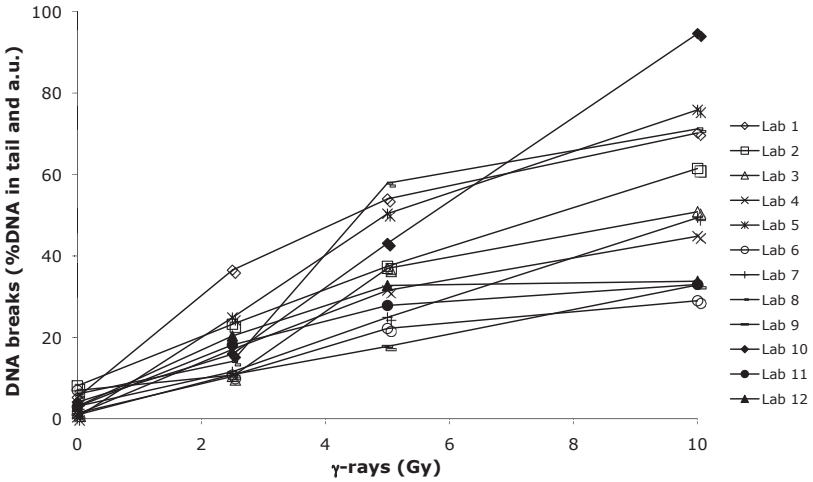

Figure 5. DNA breaks in the pre-made slides (%DNA in tail for $n = 11$ and a.u. in the scale 0-100 for $n = 1$). Each line represents the value from one laboratory.

In paper I, all participating laboratories detected the same ranking in coded THP-1 cells irradiated with 1.5, 2.5, 5 and 7 Gy of γ-radiation analysed using respective protocols. Both the dose of γ-radiation and the inter-laboratory variation contributed highly significantly ($P < 0.001$, $n = 12$) to the variation in primary comet assay endpoints (with 43.6% and 43.5% of the total variation, respectively). We showed in paper I that the inter-laboratory coefficient of variation (CV) could be significantly decreased from 47% to 28% by transforming the primary comet assay endpoints to lesions/10^6 bp by using protocol-specific calibration curves as compared to a common calibration curve (Levene's test) [69]. Adjusting primary comet assay endpoints to the own calibration curves reduced the inter-laboratory variation from 43.5% to 16.3% of the total variation and increased the contribution of the dose of γ-radiation from 43.6% to 77.1% [69]. Transformation of primary comet assay endpoint into real units by using calibration curves is further discussed in the following chapter.

Coding is recommended to avoid scoring bias. In paper I, the dose of γ-radiation contributed less to the overall variation in the coded samples than in the calibration curve samples, which was not coded [69]. However, there was no statistically significant difference between the reference samples and coded samples exposed to the same doses (2.5 and 5 Gy), indicating that knowledge about the samples did not affect the outcome [69].

4.3.3 Analysis of coded samples containing oxidatively damaged DNA

In paper II, nine out of ten participating laboratories detected the same positive ranking of the sum of DNA breaks and Fpg-sensitive sites in the coded A549 human type II alveolar epithelial cells exposed to 0, 0.2 and 0.8 μM Ro and light, once again analysed using respective protocols. Similarly, seven out of eight participating laboratories detected the dose-response when calculating the net Fpg-sensitive sites (figure 6). The used comet assay protocols significantly affected the outcome of the comet assay (discussed in the following chapter). After conversion of the primary comet assay endpoints to lesions/10^6 bp, by using the laboratory-specific calibration curves, the contribution of the dose of Ro increased from 49% to 73% of the total variation, whereas the inter-laboratory variation decreased (figures 6a and 6b).

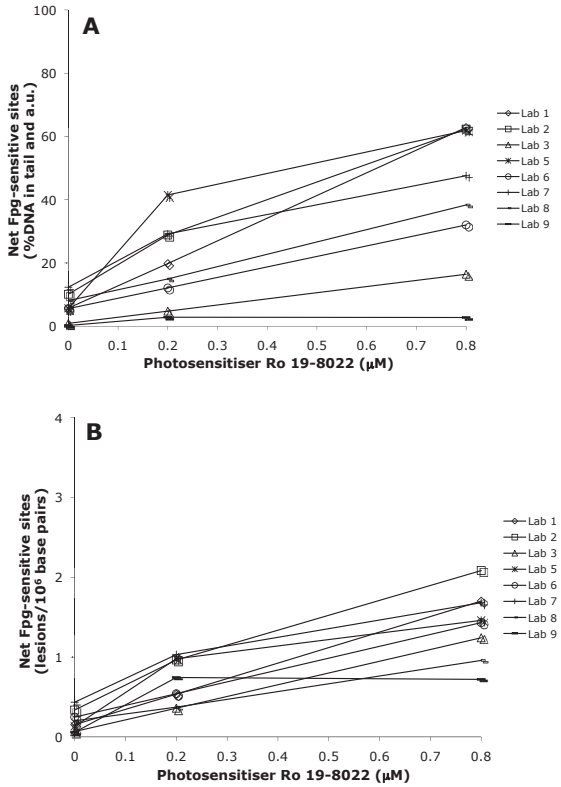

Figure 6. The level of net Fpg-sensitive sites in coded samples with A549 cells exposed to 0, 0.2 or 0.8 μM Ro and light. Eight laboratories used their own comet assay protocols to measure the net Fpg-sensitive sites. Data are presented as **(A)** %DNA in tail (n = 7) and a.u. in the scale 0-100 (n = 1), and as **(B)** lesions/10^6 bp. The fraction of explained variation strongly increased when using lesions/10^6 bp as endpoint. The amount of lesions/10^6 bp was calculated based on the assumption that 1 Gy induces 0.29 strand breaks/10^9 Dalton DNA and using laboratory-specific calibration curves.

4.4 THE ECVAG DOSE-RESPONSE, AN IMPROVEMENT SINCE ESCODD.

Regarding net Fpg-sensitive sites, seven out of eight laboratories identified this dose-response in paper II [70]. In a similar trial conducted by ESCODD published in 2003, only three out of eight laboratories could detect the dose-response relationship in net Fpg-sensitive sites using the comet assay [66]. In addition, two laboratories that used other enzymatic methods (alkaline elution and alkaline unwinding, respectively), also detected the dose-response [66]. In the ESCODD trial, HeLa cells were exposed to 0, 0.2 or 0.4 μM Ro and light for 2 min [66], whereas A549 cells were exposed to 0, 0.2 or 0.8 μM Ro and light for 5 min in paper II [70]. The wider concentration range used in the ECVAG trial might explain why we were more successful at detecting the dose-response than ESCODD.

In conclusion, the participants in ECVAG were successful in detecting dose-responses in coded samples [69]. However, although comet assay is a valuable and reliable tool to detect DNA damage in biological samples, there was considerable inter-laboratory variation due to differences in protocols and in image analysis. This is extensively discussed in the following chapter.

5 CONCERNS AND SOLUTIONS RELATED TO THE COMET ASSAY

A major concern with the comet assay is that there is no standardised protocol, and results depend to a large extent on the protocol that is used. Several guidelines for the comet assay have been published [27-29, 31], but no standardised protocol exists and there are considerable differences in those used by different research groups. These differences negatively impact the inter-laboratory comparisons of results. The results in paper I indicated that both protocol and image analysis affected the outcome of the comet assay, but no individual protocol parameters could be identified as particularly important [69]. The results in paper II, however, pointed to several important aspects in the procedures that may affect DNA migration [70]. It shall be emphasised that neither paper I, nor paper II, was designed to investigate differences in protocols as they involved too few observations with large procedural variability, which resulted in very poor statistical power. Based on the results in paper II, we decided to assess the impact of some particularly important parameters in the comet assay procedure and compile them in a methodology paper (paper III). The results from this initiative verified that the protocols clearly affect the level of DNA migration in the comet assay [72]. It wouldn't have been feasible to test all protocol variables, hence a subset was chosen, which were likely to have a large impact on the endpoint. The influence of the protocol is thoroughly discussed in the first section of this chapter. Parameters that have not been investigated in papers II or III have been observed or suggested to affect the DNA migration by others [73]. In addition, several other concerns and solutions related to the comet assay are discussed, such as the use of different units, application of reference standards and standardised protocols, range of detection of the method, enzyme specificity, and problems that need to be overcome in order to transform the comet assay to a high-throughput assay that can be used in large biomonitoring studies. In the last section of this chapter, possible interactions of particles with the enzyme Fpg are mentioned. Frequently, comet assay is said to measure DNA breaks, enzyme-sensitive sites etc, which is in a strict definition imprecise, since it is DNA migration that is measured as an estimate of different types of DNA lesions and repair. Therefore, when reporting and discussing the influence of protocol on the outcome of analysis (in this chapter and in paper III), the expression "DNA migration" was considered to be the most appropriate term to use.

5.1 THE CHOSEN COMET ASSAY PROTOCOL AFFECTS THE LEVEL OF DNA MIGRATION

Many parameters in the comet assay protocols used by different investigators differ substantially; for example, the density of the agarose gels, duration of the alkaline unwinding and electrophoresis, and strength of the electric field used. It was not within the objectives of paper II to identify what elements in the different comet assay protocols were of importance for DNA migration. The aim was simply to assess variation in estimates of oxidatively damaged DNA between different laboratories using their respective Fpg comet assay procedures. Observations suggested, however, that some protocol steps were of particular importance, i.e., agarose density, duration of enzyme treatment, extent of alkaline treatment, and time of electrophoresis, and thus we decided that it was of interest to report this, although, as emphasised in paper II, the design of the study was not optimal for such statistical evaluations. The aim in paper III was to assess the protocol parameters that seemed to affect the outcome in paper II in detail. Paper III, confirmed that **a)** there is a significant linear dose-response relationship between the agarose gel density and DNA migration, and that damaged cells are more sensitive to density variation, **b)** incubation with Fpg for 10 min is inadequate, whereas 30 min is sufficient, **c)** the typically used 20 min of alkaline treatment might be too short when analysing samples that contain particular ALS, and **d)** the duration of electrophoresis, as well as the strength of the electric field applied, affect the DNA migration [72]. The study design of paper III, with respect to the above mentioned parameters, were to a large extent based on the protocols used by the co-authors of papers I and II [69]. Also, the protocol used in the laboratory of the author of this thesis was always included. In the following section, the different parameters of the comet assay protocol are discussed with focus on those studied in papers II and III.

5.1.1 Agarose concentration

In paper II, multiple regression analyses of the influence of protocol parameters on oxidative DNA lesions indicated that the agarose density significantly contributed to the variation in DNA migration when different laboratories analysed coded samples with induced oxidative DNA lesions [70]. The agarose density was significantly correlated to the DNA migration for both the sum of strand breaks and Fpg-sensitive sites ($P < 0.001$, negative association), and the net Fpg-sensitive sites ($P < 0.05$, negative association) in a multiple regression analysis (where the level of DNA lesions expressed as %DNA in tail was the dependent variable, whereas the concentration of Ro, agarose density, duration of alkaline treatment, enzyme treatment and electrophoresis were covariate [continuous] variables) [70]. However, the ECVAG study was not designed to investigate contribution of protocols, and when the contribution of the agarose was analysed in a model solely with the concentration of Ro, it did not

significantly affect the variation in Fpg-sensitive sites [70]. Although it may seem trivial to delve into agarose concentration and DNA migration, no thorough investigation of the impact of this parameter in the alkaline comet assay protocols had been performed (published) as far as we were aware at that time. Several articles had referred to Tice [23], stating that agarose concentration affects the outcome, but no data was presented. We showed in paper III (figure 7) that there is a significant linear dose-response between the agarose gel density and DNA migration, which is in accordance with recent findings by Azqueta *et al.* [72, 74]. A similar pattern was observed by Bauch *et al.*, who observed that by increasing the agarose concentration from 0.5 to 1% and from 0.5 to 1.5%, the %DNA in tail was reduced by a factor of 2 and 3, respectively, using a neutral comet assay protocol [75]. In paper III, cells exposed to 5 Gy of γ-radiation seemed to be more sensitive to alterations in agarose density than untreated cells (figure 7), indicating that the sensitivity of the comet assay can be modified by altering agarose concentration [72]. However, Azqueta *et al.* recently concluded that a final agarose concentration between 0.6 and 0.8% seems to be optimal to avoid unstable gels and impeded DNA migration [74]. Even though Bauch *et al.* used a different comet assay protocol than the one employed in the papers in this thesis, they came to the conclusion that 0.75% agarose was most favourable [75]. Agarose concentrations within this range are used by many laboratories, and by the author of this thesis (final agarose concentration of 0.7% is used). Taken together, these findings highlight the importance of keeping the agarose concentration constant during a study, i.e. to handle the agarose with care and prepare the agarose solution in a strict manner. When pipetting agarose solutions, its viscosity should be considered. In addition, storing the agarose in small aliquots is recommended since repeated melting can affect the density by evaporation [74].

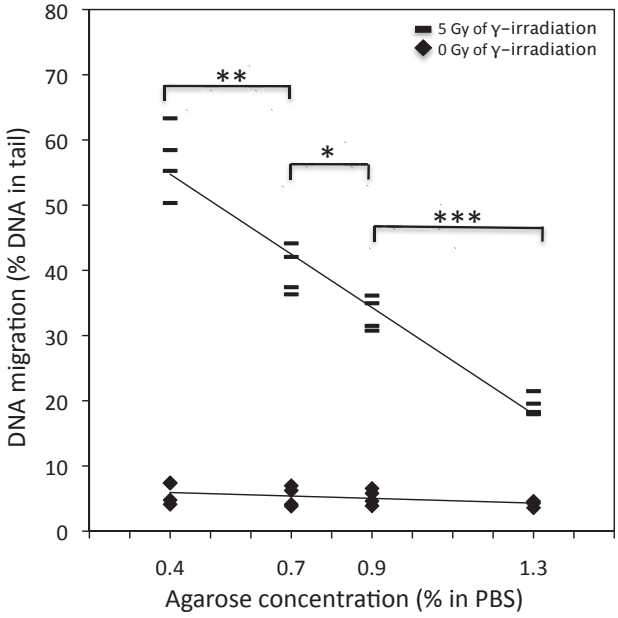

Figure 7. DNA migration in untreated cells i.e. cells exposed to 0 Gy (filled diamonds) and cells exposed to 5 Gy (lines) of γ-radiation measured by the alkaline comet assay using different agarose densities. Each increase in agarose density caused a significantly decreased DNA migration in cells irradiated with 5 Gy (Student's t-test, where *, ** and *** means $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively). The linear dose-response relationships were significant both for untreated cells ($r = -0.71$, $P < 0.01$, ANOVA) and cells exposed to 5 Gy of γ-radiation ($r = -0.95$, $P < 0.001$, ANOVA). The slopes of the two regression curves were significantly different ($P < 0.001$) from each other. The experiment was repeated 4 times. Each diamond/line represent the mean value of 100 scored cells (50 cells from each of two separate gels).

An interesting observation within the ECVAG network was that many participants reported the agarose concentration before adding the PBS-cell suspension, instead of the *final* agarose concentration. When we specified that the participants should report the *final* agarose concentration in the second round of studies (ECVAG studies 5-7 described in section 4.2), the results were as expected different. It shall be highlighted that it is the real *final* agarose concentration that is most informative to other scientists.

5.1.2 Lysis

The duration of the lysis treatment is widely believed not to influence the outcome of the comet assay and differs substantially between different laboratories (often ranging from 1h to overnight). A warning is issued in the '*NewGeneris Comet Assay Protocols and other useful information*' [76], regarding that a longer lysis treatment can cause detachment of gels. In ECVAG study 5, a long lysis treatment caused detachment of gels for several participants (Forchhammer *et al*., in preparation).

To further avoid possible detrimental factors, Hartmann *et al.* recommend rinsing of the slides before alkaline treatment to remove remaining constituents from the lysis solution that may influence the electrophoresis [29].

5.1.3 Incubation time of Fpg and hOgg1

Before using an enzyme in the comet assay, the investigator should perform a titration experiment where the saturation level is assessed by varying the enzyme concentration and/or the enzyme treatment time [28]. Results in the literature and observations in paper II show that investigators at times use far too short enzyme incubation [70]. The durations of Fpg treatment in paper III were based on the durations used by the participants in ECVAG in paper II, where incubation with Fpg was performed for 10 min (n = 1), for 25 min (n = 1), for 30 min (n = 5), and for 45 min (n = 3) [70]. The results indicated that incubation with Fpg for 10 min is inadequate whereas 30 min is sufficient to introduce the additional breaks in the DNA [72]. Dušinská & Collins suggested that an incubation of 30 min appeared optimal already in the first Fpg comet assay paper [10]. The Fpg used in papers II and III, and by many other researchers, is of one batch and concentration, and obtained from A.R. Collins (University of Oslo, Norway). Based on this, it can be argued that it is useful to some researchers to highlight the importance of the duration of Fpg treatment. Whereas the quality of the commercially available Fpg varied a lot in our hands, the enzyme produced by A.R. Collins proved to be more stable. Based on unpublished data from J. Kain, H.L. Karlsson and L. Möller (Kain, personal communication) it can be speculated that the durations of incubation with hOgg1 presented in some publications might be too short. Although Fpg recognises a range of additional substrates *in vitro* compared to hOggI, it remains to be seen how biologically important and abundant these other lesions are *in vivo*. A systematic comparison of the background levels of hOgg1- and Fpg-sensitive sites in a large biomonitoring trial has not been performed to the knowledge of the author of this thesis [77].

The availability of the enzyme is an important aspect for the applicability to the comet assay. It is an advantage if the enzyme can be produced through an over-producing

plasmid or phage DNA, allowing for mass production of the enzyme by bacteria in a cheap and simple way. The use of hOgg1 in the comet assay is far less common as compared to Fpg, as it is not available in large quantities [28] and has poor stability in the freezer.

5.1.4 Duration of alkaline unwinding and its significance for ALS

At pH>12.6, ALS are believed to be transformed to strand breaks quickly [78]. Tice *et al.* stated in 2000 that 20 min alkaline unwinding is considered sufficient for most purposes [31]. However, several papers have reported that the DNA migration is affected by the duration of alkaline unwinding [79, 80] and Collins points out that it has not been rigorously shown that all ALS are converted to strand breaks in the comet assay [81]. When we analysed the influence on DNA migration by the concentration of Ro and the different protocol-steps in paper II, the duration of alkaline treatment significantly contributed to both the sum of DNA breaks and Fpg-sensitive sites ($P < 0.01$), and net Fpg-sensitive sites ($P < 0.01$) [70]. The statistical significance remained when using a statistical model with solely the concentration of Ro and the duration of alkaline treatment [70]. Typically, 20-40 min of alkaline unwinding is applied [70], but 60 min alkaline unwinding was also included in paper III, since findings by Vijalaxmi *et al.* indicated that there is an increased level of DNA migration after 60 min alkaline treatment in cells exposed to γ-radiation [80]. Similarly, we observed in paper III that the duration of the alkaline unwinding (20, 40 and 60 min, respectively) affected the extent of the DNA migration in cells exposed to 5 Gy of γ-radiation [72]. In cells exposed to Ro and light, which is believed to mainly induce 8-oxoguanine [82], there was a significant difference in DNA migration between cells exposed to 40 and 60 min of alkaline unwinding [72]. In H_2O_2-treated cells, however, we observed a significant difference in DNA migration when prolonging the alkaline unwinding from 20 to 40 min, whereas increasing the time an additional 20 min to 60 min did not affect the outcome significantly (Student's t-test) [72], which is in accordance with findings by Azqueta *et al.* [74]. We did, however, observe a highly significant dose-response relationship between 20-60 min alkaline unwinding (ANOVA) in cells exposed to both H_2O_2 and 5 Gy, as well as a significant dose-response relationship in cells exposed to Ro and light [72]. The DNA migration in undamaged cells was not affected by the duration of alkaline treatment [72]. Taken together, these results indicate that cells exposed to γ-radiation, Ro and H_2O_2 contain ALS, and that not all of these ALS have been converted to strand breaks after 20 or 40 min of alkaline treatment [72]. The significantly different slopes of the untreated and treated cells indicate that the sensitivity of the method could be improved by altering the duration of the alkaline treatment [72]. Typically used durations of alkaline treatment might be inadequate, provided that one *wants* to measure these ALS. Speit & Hartmann suggested that durations of alkaline treatment and

electrophoresis should be adjusted to obtain valid and reproducible results for each cell type [83]. We do not know the nature of these ALS that seem to be resistant to alkaline treatment, nor whether they are frequently occurring or whether it is of significance to measure them.

5.1.5 Electrophoresis

5.1.5.1 Strength of the electric field

It is commonly known that the strength of the electric field (the electric potential) affects DNA migration in a linear way during electrophoresis. However, due to the particular circumstances in the comet assay, where strand breaks allow relaxation of DNA loops and allow them to extend towards the anode, this is not necessarily true for the comet assay. Without strand breaks there is hardily any DNA migration irrespective of the sensitivity of the protocol. We observed in paper III that the strength of the electric field did not have a statistically significant impact on cells exposed to 0 Gy (untreated cells) (figure 8) [72]. When damage is present, on the other hand, significantly different slopes of the regression curves for the different strengths of the electric field indicate that the sensitivity of the comet assay can be improved by altering the strength of the electric field (figure 8) [72]. However, applying a too weak or a too strong electric field is not recommended. Applying an electric potential of 0.70 V/cm in paper III resulted in very short comet tails in all cells. Azqueta *et al*. recently demonstrated that H_2O_2-treated cells behave like untreated cells when an electric potential of 0.16 V/cm is applied, i.e. there is virtually no migration at all [74]. Thus, it seems that when the strength of the electric field is insufficient, the DNA does not migrate, i.e. some DNA loops have not extended out from the comet head resulting in an underestimation of DNA damage. An electric potential of 1.60 V/cm, on the other hand, resulted in very long comet tails, which were difficult to score due to difficulties defining the comets head and tail [72]. Azqueta *et al.* faced the same scoring difficulties when analysing H_2O_2-treated cells using an electric potential of 1.48 V/cm, and described it as comet tails being detached from the comet heads [74]. When a large fraction of the DNA is in the comet tail, the DNA migration could end up outside the range of linear detection, i.e. not more than ~80% of the DNA should be in the tail [84]. When applying 1.60 V/cm in the analysis of cells irradiated with 7.5 Gy of γ-radiation in paper III, the range of linear detection was approached (figure 8) [72].

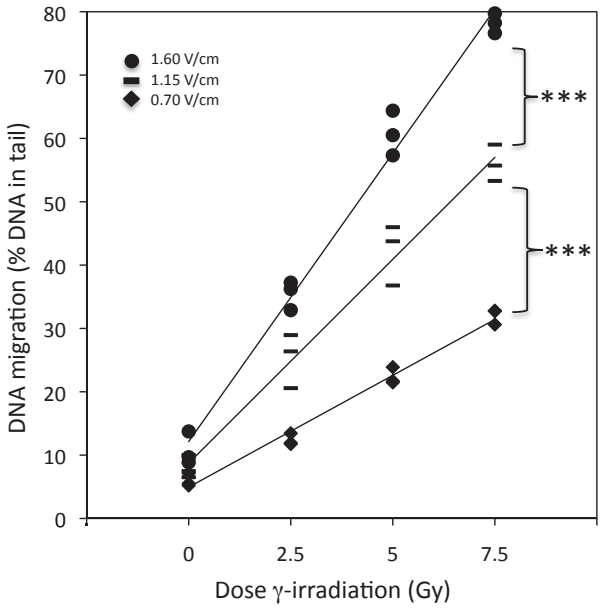

Figure 8. DNA migration in cells irradiated with different doses of γ-radiation measured with the alkaline comet assay using different strengths of the electric field (0.70, 1.15 and 1.60 V/cm) during electrophoresis. The DNA migration was significantly increased ($P < 0.001$, two-way ANOVA) when comparing 1.60 V/cm (closed circles) to 1.15 V/cm (lines) and when comparing 1.15 V/cm to 0.70 V/cm (filled diamonds) and the slopes of the regression curves were significantly different from each other ($P < 0.001$). The linear dose-response relationship ($r = 0.99$ for all strengths of the electric fields) was highly significant for all curves ($P < 0.001$, ANOVA). The experiment was repeated 3 times. Each diamond/line/circle represents the mean value of 100 scored cells (50 cells from each of two separate gels).

In the '*NewGeneris Comet Assay Protocols and other useful information*', it is suggested as a rough guide to use about 1 V/cm with a layer of 1-2 mm of buffer above the slides [76]. Although a linear dose-response relationship was observed for the electric field when investigating 0.70, 1.15 and 1.60 V/cm in paper III (figure 8), one should be careful to draw any general conclusions regarding a linear dose-response over this entire interval. There is not necessarily a linear dose-response relationship between for example 1-1.2 V/cm.

Ampere (A) or voltage (V) is often reported in scientific papers when describing the strength of the electric field used in the comet assay. However, since there is a wide

range of different electrophoresis tanks with different sizes and proportions available, this is quite uninformative. It is more accurate to report the voltage gradient over DNA (V/cm) since it is this gradient that causes the DNA to migrate [26]. Prof. G. Brunborg (National Institute of Public Health, Oslo, Norway) has prepared an Excel sheet (available online) that returns the V/cm based on the physical dimensions of the tank (inner dimensions and length of platform), the power supply (voltage and current) and buffer characteristics (amount, conductivity, pH, temperature and molarity of the buffer) [85]. This Excel sheet was used in papers I and II, albeit some designs of the electrophoresis tanks were more difficult to apply to the data sheet than others. It is a good approximation to divide the voltage with the length of the platform in the electrophoresis tank since the voltage drop between the platform and the electrodes is very small compared to above the platform [26]. During the preparation of papers I and II it was discovered that there was a lack of consensus regarding how to calculate the voltage gradient and it seems to be a common misunderstanding that the V/cm should be based on the distance from the anode to the cathode. Investigators should make sure that the voltage gradient is kept constant during a study. In a recent article by Azqueta *et al.,* the authors question the accuracy of calculating V/cm both across the platform and from anode to cathode [74]. By measuring the actual voltage potential on the platform (by a jig equipped with platinum probes set 10 cm apart on, and 0.7 mm above the platform) the authors demonstrated that the real voltage potential differed substantially from the V/cm calculated from both the anode to cathode and across the platform [74]. This leaves an even greater confusion about how to report the electric potential. Due to the confusion regarding V/cm, and to the great diversity of the physical dimensions of the electrophoresis tanks used by the different ECVAG collaborators, we chose not to include the strength of the electric field in the multiple regression models in paper II.

5.1.5.2 Duration of Electrophoresis

The duration of electrophoresis affected both the sum of DNA breaks and Fpg-sensitive sites ($P < 0.01$) and the net Fpg-sensitive sites ($P < 0.001$) in paper II (multiple regression analysis) [70]. This supported previous findings by Forchhammer *et al.* [79]. Again, in paper III, it was shown that the duration of the electrophoresis significantly affected the outcome of the comet assay (figure 9) [72]. It is possible that the significantly steeper slope in figure 9 reflects an increased level of converted ALS into strand breaks in the cells that have been irradiated to higher doses of ionising radiation. Since electrophoresis is performed in the same alkaline solution as alkali unwinding, it can be speculated that the increased duration of alkaline treatment affects the outcome more in samples that contain more ALS relatively resistant to alkaline treatment.

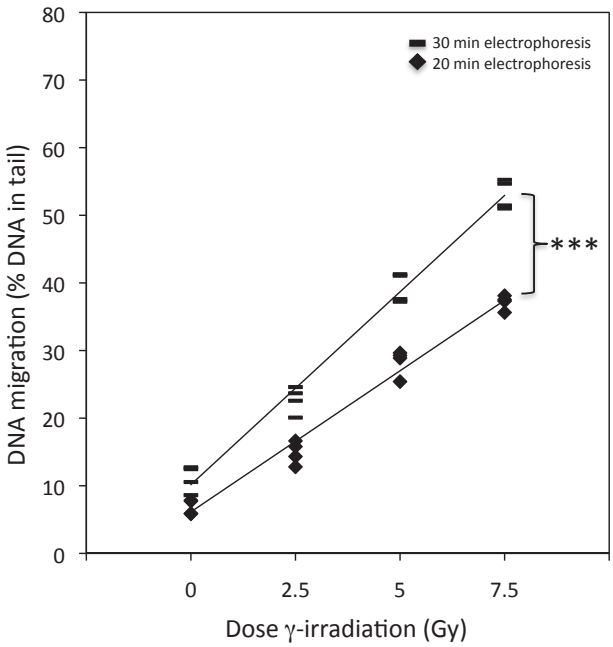

Figure 9. DNA migration in cells irradiated with different doses of γ-radiation measured with the alkaline comet assay with 20 or 30 min of electrophoresis. The DNA migration was significantly increased ($P < 0.001$, two-way ANOVA) when running electrophoresis for 30 min (lines) compared to for 20 min (filled diamonds), and the slopes of the two regression curves were significantly different from each other. The linear dose-response relationships for both curves were highly significant ($r = 0.99$, $P < 0.001$, ANOVA). The experiment was repeated 4 times. Each diamond/line represent the mean value of 100 scored cells (50 cells from each of two separate gels).

5.1.6 Scoring of comets

The relative magnitude of the intensity of the comet tail provides information about the number of relaxed loops, which reflect the level of DNA damage [42, 67]. The comets are analysed either by visual or computerised scoring. The different ways of scoring cells have different advantages and weaknesses. Visual scoring and computerised scoring have been shown to correlate to each other [8, 58, 86]. The recently developed automatic computerised scoring is completely automatic compared to the time-consuming computerised analysis, which requires that an investigator watches and selects the comets to be analysed. Azqueta *et al.* recently compared visual scoring, computerised scoring and completely automatic computerised scoring and concluded that all three

scoring methods are thrust-worthy and that results obtained by the three methods to a large extent can be interchangeable [54]. Advantages with visual scoring are that it is faster than computerised scoring (when performed by an experienced investigator) and that cells in close proximity to each other can be scored. Drawbacks of visual scoring are that the method suffers from overestimation of low levels of damage [54] and that different investigators have reported different levels of DNA migration when scoring the same slides [87, 88]. However, in a study by Møller *et al.,* it was clear that the investigators with most experience showed the lowest levels of variation [88]. In this context, it should be emphasised that the participants in ECVAG reported substantially different levels of DNA migration (%DNA in tail) in the pre-made slides in paper I using computerised image analysis [69]. Although these pre-made slides were not the same set of re-stained slides, they were prepared in the same laboratory using a fixed protocol.

When working with computerised scoring, it is important that the density of cells in the gels is appropriate. Cells shall neither be 'selected' nor 'excluded' by the investigator. A high density can give artefacts since overlapping cells or cells in close proximity cannot be scored (causing a 'selection'), whereas a low density will make the scoring time-consuming and difficult. One should make sure that the same cell is not scored more than once. This can be achieved for example by scoring all cells encountered in 'a row in the gel' until a specific amount of cells has been scored. When using computerised scoring, the software program recognise the head and the tail of the comet, but the investigator can change the borders of the head and tail if the program fails to do so properly (which is not uncommon). Also, when a cell in close proximity falls into another cell's 'background', the investigator can adjust the microscope slide to enable the comet to be scored. Investigators have noted that comets located in the outer areas of the agarose gel are sometimes more damaged and in order to avoid edge effects, it is better to avoid scoring too close to the gel edges.

The findings in paper I imply that the image analysis (and possibly staining) affects the outcome of the comet assay [69]. We suspect that this is related to different settings in the image analysis system. However, it cannot be completely excluded that different stains have affected the outcome. For example, EtBr intercalates between base pairs whereas DAPI binds in the minor groove [26], which possibly might affect the relative intensity between the comet head and comet tail, since the latter consists of more single-stranded DNA. Also, stains differ in photostability [75]. In study 5-7 in ECVAG, this question was addressed by distributing pictures of different classes of comets together with information on approximate values of %DNA in the tails. The participants were instructed to change the settings of their image analysis software if the results deviated from these images. However, it can be expected that investigators will be reluctant to

change such settings, especially if originally adjusted by manufacturers to optimal conditions. Taken together, there were too many different image analysis programs and stains used to statistically verify their influence in the ECVAG trials.

5.1.7 Used solutions/buffers

It is clear that the alkaline (pH>13), the low alkali (pH~12.3), and the neutral comet assay affect the endpoint. However, as observed in paper II, most researchers now use similar solutions in the alkaline comet assay, e.g. lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10), Fpg enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0) and alkaline solution (0.3 M NaOH, 1 mM EDTA, pH>13). Some of the participants in ECVAG have reported that their alkaline buffer had pH=13 and some reported that it had pH>13. Many pH meters are not calibrated to measure such a high pH, and since the same alkaline solution (0.3 M NaOH, 1 mM EDTA) was used by participants, it was assumed that the pH was the same in all laboratories in papers I and II.

5.1.8 The influence of temperature

Speit *et al.* observed that the duration of alkaline treatment and electrophoresis time influenced the DNA migration, but also that the employed temperature drastically affected the outcome [73]. When comparing 4°C with room temperature (20°C), a substantial increase of DNA migration was seen at room temperature suggesting that temperature is a major parameter affecting the sensitivity of the comet assay [73]. The authors concluded that the assay has to be performed under constant and reproducible conditions [73]. In the laboratory of this author, this is achieved by always performing lysis and alkaline treatment at 4°C and by employing an electrophoresis tank that is chilled by ice-cold water circulating under the platform. Analysis performed at room temperature would be made difficult due to temperature variations during seasonal changes.

5.2 UNITS

The most frequently used units for computerised scoring are %DNA in tail, tail moment and tail length. Collins *et al.* have recommended the use of %DNA in tail, since it is linearly related to break frequency over the widest range of damage and linearity is needed in order to calculate net enzyme-sensitive sites [26]. Tail length is linear at low levels of damage [89] but a drawback is that it is only linear in a very narrow interval [9]. Collins *et al.* also argues that the tail length can differ between species and tissues [26]. We observed in paper I that tail length was a poor unit that did not even allow for detection of dose-response in the pre-made slides [69]. A difficulty with tail moment

is that it can be calculated in different ways, e.g. 'tail extent moment' being the product of %DNA in tail and the tail length, or the 'olive tail moment' being the product of %DNA in tail and the distance of centre of gravity between head and tail [90, 91]. In both cases, tail moment is based on tail length, so a deviance from linearity in tail length will affect the linearity of tail moment as well. The dose-response relationships in the pre-made slides obtained by %DNA in tail or tail moment did not differ significantly from each other in paper I [69].

An observation within ECVAG is that scientists seem to have rather strong opinions regarding whether it is more appropriate to report %DNA in tail as a median or mean value. The distribution of damage in γ-irradiated cells is homogenous, whereas (for example) exposure to particles can lead to a heterogeneous distribution i.e. some cells might be undamaged whereas some are very damaged [92]. In the latter case, a 'median' value, could conceal the effect. On the other hand, Speit & Hartmann suggest that since the damage levels in the individual cells are frequently not normally distributed, median is the preferred unit [83]. 'Mean' might give excessive weight to heavily damaged comets, e.g. when most cells have 5% of the DNA in the tail except one or a few comets that have 95% of the DNA in the tail. Also, some cell types might have more heterogeneous distributions than others. In experience of this author, cultured cells frequently have a more homogenous distribution than PBMC. In addition, H_2O_2-treatment of lymphocytes results in a relatively heterogeneous distribution of damage [54]. One reason for this heterogeneous result could be that PBMC are frequently isolated using gradients and PBMC isolated in this manner consist of different cell-types (but mainly lymphocytes). Cells of for example different age or in different stages of the cell cycle can also respond differently. Azqueta *et al*. recommends that median should be used for most comet results [54]. When the level of damage in the analysed cells is very heterogeneous, it can for example be appropriate to report results as distribution patterns using histograms [93]. Experienced scientists are likely to be able to judge which endpoint/endpoints (mean, median or a distribution pattern) are most appropriate for a particular dataset. The possibility to measure heterogeneity in response (to detect the response in individual cells) is an advantage with the comet assay, and it is important not to loose valuable information when reporting data.

5.3 REFERENCE STANDARDS

Experimental variation can be decreased by calibration with ionising radiation or by using internal standards.

5.3.1 Calibration curves

By calibration with γ-rays or X-rays, primary comet assay endpoints, e.g. %DNA in tail, tail moment and arbitrary units, can be reported in 'real' damage levels, e.g. lesions/10^6 bp, lesions/10^9 Dalton DNA, lesions/cell or Fpg-sensitive sites/10^6 guanines. Before starting to discuss the problems with calibration curves, it should be stressed, nonetheless, that calibration by protocol-specific calibration curves is a powerful and useful tool with which to reduce variation [72]. In addition to facilitating comparisons between studies that have used different protocols, 'real' units is easier to understand for individuals who are not familiar with the comet assay compared to primary comet assay endpoints. The benefits of calibration are easy to understand, but there are also some methodological problems that should be mentioned. Methodological difficulties with standard curves include; **a)** it is laborious, **b)** an ionising radiation source is required, **c)** it is possible that the type, energy and quality of the radiation source can affect the outcome, **d)** repair (or induction) of DNA-damage should be prevented, and, **e)** the accuracy of the assumed amount of DNA breaks that is formed per Gy might be questioned. Also, in papers I and II, it was observed that there was a tendency to approach saturation at 10 Gy, seen as deviation from linearity for some laboratories at this radiation dose, which indicated that the upper limit of detection had been reached. This is in accordance with observations by others [94]. The sensitivity of the respective protocols should to be taken into consideration when selecting the dose-range of the calibration curve. In the ECVAG studies 4-7, the dose range used in the calibration curves was modified to 0-7.5 Gy.

Including reference curve standard samples is laborious, and since the limitation of the comet assay often is the size of the electrophoresis tank, including reference standards might mean that some other important samples have to be excluded. However, the emerging high-throughput systems using mini-gels and automatic scoring minimise this problem.

Differences in calibration curves between laboratories, i.e. in the level of DNA migration caused per Gy, have been highlighted before, and this has been suggested to depend on protocol differences and the use of different radiation sources, which can differ in type, energy and quality [26]. Also, strand breaks can be repaired quickly, and the handling of cells until lysis, where all repair is terminated, has been shown to affect the standard curves [26]. Exposure to radiation (and other substances) after embedding the cells in gels can be used to better prevent repair [26]. For PBMC, DNA damage can be induced or repaired during isolation of PBMC (during collection, transportation, centrifugation and washing) or during the handling of cells until lysis (during thawing, centrifugation, washing and gel preparation). In papers I and II, where cell cultures

were used and one laboratory prepared and froze the samples, it is likely that the differences that were observed were mostly attributed to differences in protocols and settings in the analysis systems [69], but they could also in part be due to different procedures for thawing and washing. It was observed in papers I-III that the calibration curves did not completely remove the influence of the different protocols [72]. In paper III, we speculate that relative amounts of different types of damage influence samples' sensitivity to duration of alkaline treatment and electrophoresis. Already in 1992, Vijayalaxmi *et al.* speculated that γ-irradiated cells contain ALS that are relatively resistant to high alkaline treatment [80]. The results in paper III indicate that the typically applied 20 or 40 min of alkaline treatment might not be sufficient to convert all ALS into strand breaks in γ-irradiated cells. Calibration curve samples that contain ALS that are relatively resistant to alkaline treatment will make the accurateness of the transformation of damage levels into 'real units' vulnerable to the length of alkaline treatment and electrophoresis. The samples that one wants to investigate might also contain these types of ALS making them sensitive to the length of the alkaline treatment and electrophoresis.

5.3.2 Transformation of units in papers I-III

When converting DNA damage into 'real units', assumptions are made regarding the amount of strand breaks caused per Gy of ionising radiation. When converting %DNA in tail and a.u. into real strand breaks in the ECVAG studies, an average of two estimations of the amount of strand breaks that is caused per Gy was used. The amount of strand breaks per 10^9 Dalton DNA caused per Gy was estimated to be 0.27 by Kohn *et al.* [95] and 0.31 by Ahnström & Erixon [96], using the alkaline sucrose sedimentation technique. In the ESCODD studies, the conversion was based only on the latter estimation. However, the difference between these assumptions should not be exaggerated, and it was actually shown in paper I that ESCODD's calibration curve and an average of the ECVAG participants' calibration curves were virtually the same [69]. However, it is worth noting that there is different ways to estimate 'real' units of damage. Also worth noting is that not all conversions are based on standard curves performed by the 'own' protocol [88]. Because of substantial influence on DNA migration by protocol (paper III), and that conversion of primary comet assay endpoints by 'own' calibration curves are superior to a common calibration curve (paper I), i.e. using the 'own' calibration curves decreased the inter-laboratory CV from 47% to 28%, it is strongly recommended to use a standard curve performed with the 'own' protocol when converting primary comet assay units. The formula that was used to transform the DNA migration into real units in papers I-III is described in papers I and II [70].

The two cell lines used in papers II-III (A549 and HTP-1) are of different genome size. To use different cell lines was originally a mistake, and the issue has been extensively discussed within ECVAG. Based on the partners' past experience, the genome size of the irradiated cells does not affect the DNA migration (%DNA in tail) since irradiation 'saturates the DNA', e.g. causes 0.29 lesions/10^9 Dalton DNA, disregarding of the amount of DNA. Therefore, it was decided that the conversion into real units should not take the genome size, e.g. the different cell types, into account.

5.3.3 True internal standard

An alternative to calibration curves is using internal reference cells. Zainol *et al.* (study 4 in ECVAG) have developed distinguishable reference cells that can be embedded in the same gels as the investigated test cells [71]. By replacement of thymidine by bromodeoxyuridine, and applying a fluorescently tagged anti-bromodeoxyuridine antibody together with an additional filter, the reference cells can be distinguished from the test cells [71]. Zainol *et al.* showed that the intra-experimental and inter-experimental variation was greatly improved by using these internal standards and that this effect partly was attributed to the correction of inter-gel variability [71].

5.4 STANDARDISED PROTOCOL

Whether results in different studies could be directly compared when a standardised protocol is used needs to be elucidated. In ECVAG study 5, a set of reference conditions will enable comparisons between laboratories without the impact of some particularly critical protocol differences (Forchhammer *et al.,* in preparation). Preliminary statistical analyses from ECVAG study 5 indicate that it is possible to decrease, but not completely remove, the inter-laboratory variation by using reference conditions, i.e. fixed solutions, agarose concentration, enzyme conditions, duration of alkaline treatment, duration of electrophoresis and strength of the electric field (Forchhammer *et al.,* in preparation). However, it should be emphasised that only a few protocol parameters were standardised in the ECVAG study 5, and the reference conditions cannot be considered to be a standardised protocol.

It is unlikely that investigators could be persuaded to change their own procedures radically and start to use a standardised protocol. In addition, although the lack of a standardised protocol often is regarded as a problem, it can also be an advantage for the experienced investigator that the protocol can be adjusted to improve sensitivity or to suit the composition of different DNA lesions or cell types containing DNA with different compactness. The compactness of DNA differs between cells. Cell types that are exposed to a harsh environment have a more compact DNA. Sperm cells, for example, have a six times more compact DNA than somatic cells [38].

5.5 RANGE OF DETECTION

Even though the range of detection in the comet assay is quite narrow, it is very well suited for investigating normal background levels of DNA damage and damage caused by non-lethal exposure to different compounds (approximately a hundred to several thousand breaks) [26]. The upper limit of the comet assay is due to that once all DNA is in the tail the method is saturated. But even before this occurs, there is a deviation from linearity. The comet assay is linear up to approximately 80 %DNA in tail [84]. DNA break frequency is linear to %DNA in tail up to approximately 2.5 breaks per 10^9 Dalton DNA [9]. When using enzymes in the comet assay for simultaneous measurement of DNA breaks and enzyme-sensitive sites, it is important that the saturation level for the sum of DNA breaks and enzyme-sensitive sites is not reached in order for the net enzyme-sites to be correct, i.e. linearity is required for correct calculations of net enzyme-sensitive sites. Comet assay's detection limit for ionising radiation has been reported to be as low as 0.05 Gy [89] and 0.006 Gy [97]. Based on the assumption that 1160 strand breaks are created per Gy in diploid cells, this would correspond to 58 and 7 strand breaks, respectively.

5.6 SPECIFICITY OF THE USED REPAIR ENZYMES

The enzyme comet assay is often regarded as being less specific when measuring oxidative DNA lesions than HPLC based techniques due to that the used enzymes (such as Fpg) recognise a range of different damages. However, ESCODD showed that comet assay is superior at measuring oxidative DNA lesions compared to both HPLC based techniques and GC-MS techniques [67]. One should keep in mind, however, that the enzymes generally recognise several other lesions in addition to their main substrate (described in section 3.2.4). For example, Ro and light, which was used in both ESCODD and in papers II and III, is assumed to mainly induce 8-oxoguanine. Pflaum *et al.* treated phage DNA with Ro and estimated that 74% of the Fpg-sensitive sites were 8-oxoguanine by using HPLC and the Fpg alkaline elution method [98]. In a similar way, Will *et al.* estimated that 69% of the Fpg-sensitive sites caused by Ro and light were 8-oxoguanine [82]. Ro predominantly produces singlet oxygen ($^1O_2$), which could be expected to generate relatively few DNA breaks [66, 82]. Thus, the Fpg-sensitive sites detected in papers II and III is likely to include other oxidatively modified DNA-bases in addition to 8-oxoguanine. The observed DNA breaks caused by exposure to Ro in paper II could reflect intermediate stages of repair of oxidative DNA damage.

5.7 SPECIFICITY OF THE COMET ASSAY

Do we know what we measure with the comet assay? This question can be interpreted in several ways, and different aspects of the specificity of the comet assay have already been mentioned. Different types of the comet assay clearly measure different lesions, e.g. the neutral comet assay measures strand breaks, whereas the alkaline comet assay measures strand breaks and ALS. In the enzyme versions of the comet assay, the applied enzymes frequently recognise a main substrate and lower levels of a range of other lesions (section 3.2.4). New base damages recognised by the applied enzymes will most likely continue to be discovered. It is also worth noting that although the basic alkaline comet assay is most frequently used, only a few genotoxic agents induce DNA strand breaks directly. Strand breaks are, however, often formed as intermediates during the repair process of other DNA lesions [30]. Apurinic and apyrimidinic (AP) sites formed as repair intermediates are alkali-labile, but it has not been established whether the alkaline treatment will cause a conversion of all AP sites to strand breaks [28]. AP sites are an intermediate during BER but can also be formed by other means. Different agents cause different types or patterns of DNA damage and it is important to use appropriate methods to assess the DNA damage.

In the past, the comet-tail was often said to consist of DNA fragments, whereas nowadays it is often said to consist of relaxed DNA loops. Observations that the comet-tail is often of a particular length (75 μm), which is in accordance with the estimated loop length of 220,000 bp, support the loop theory [26]. A break in a DNA loop will relax the supercoiling and allow the loop to migrate towards the anode. However, the loop theory does not exclude that the comet-tail also consists of DNA fragments, created by several breaks in a loop. Very long tails and tails that are detached from the comet head observed by Ersson & Möller [72] and Azqueta *et al.* [74], indicate that the comet tail also consist of DNA fragments.

PBMC is extensively used in studies employing the comet assay due to availability, but they are not targets for cancer. Since they circulate through the body they are assumed to reflect the organism's overall state [26]. However, whether it is appropriate to draw conclusions regarding tissues where cancer often develops from PBMC is a valid question. We observed in a recent study on salivary glands in patients with chronic kidney disease (CKD) that tissues and circulating cells seem to be differently affected in this patient group [99], although one should be careful to draw any general conclusions from this patient group. The internal environment in these patients is in many ways extreme and for those patients who undergo haemodialysis (HD), PBMC are forced through a dialysis machine exposing them to additional stress.

5.8 TRANSFORMING THE COMET ASSAY INTO A HIGH-THROUGHPUT ASSAY

The normal alkaline comet assay protocols currently employed is too laborious to be applicable to large studies with hundreds or thousands of samples. High-throughput protocols are needed in order to make the comet assay suitable for large biomonitoring studies and to study effects of for example single nucleotide polymorphisms (SNPs) [81]. The most important limitations are probably the time-consuming scoring and the size of the electrophoresis tank, which limit the number of slides that can be analysed at each analysis occasion. The network for comet assay and cell array for fast and efficient genotoxicity testing (COMICS) is developing a high-throughput protocol of the comet assay using mini-gels and completely automatic computerised scoring. Automatic computerised scoring have been shown to be equally good at finding low levels of damage as computerised scoring [54]. However, the automatic scoring program have difficulties to find heavily damaged comets [54]. Although completely computerised automatic scoring is a very promising tool, general applicability is prevented by the program's price tag.

Conventional microscope slides usually contain 1-3 gels per slide. Shaposhnikov *et al.* have developed a custom-made silicone gasket, which allow incubation of 12 mini-gels per slide with different solutions [37]. In this way, either substantially more samples, or samples incubated with several enzymes simultaneously, can be analysed in a single experiment [37]. The authors showed that results obtained by using 12 mini-gels per slide were comparable to results obtained by using two gels per slide [37]. The mini-gel system allows simultaneous electrophoresis of more samples, but it also saves time at all stages during the assay since less slides need to be handled and less reagents and solutions are needed [37].

5.9 POSSIBLE INTERACTION BETWEEN PARTICLES AND THE COMET ASSAY

When analysing DNA damage caused by particles with the comet assay, investigators have found clearly visible particles in the nucleoid, although no such particles could be seen in the cell nuclei when using transmission electron microscopy [92, 100, 101]. Karlsson states that it seems like intracellular particles can gain access to DNA after lysis when performing the comet assay [100]. Recent findings by Kain *et al.* indicate that several particle types interact with the repair enzyme Fpg during the comet assay (Kain *et al.,* in preparation). The nature of this interaction is a matter of speculation. Fpg is a so-called zinc finger protein, and the active site of the Fpg contains a zinc atom surrounded by four SH-groups. Some metal ions such as cadmium(II) and cupper(II) have been observed to be able to inhibit the enzyme's activity, as it seems by

competing with zinc for its position in the active site [102]. Kain *et al.* observed that a range of metals caused a decreased enzyme activity and that silver completely inactivated the enzyme, and suggest that silver particles or ions might bind to the SH-groups in the active site (Kain *et al.,* in preparation). Fpg is probably not the only repair enzyme sensitive to metals. Several metals including cadmium, manganese, arsenic, chromium and lead have been reported to decrease Ogg1 activity although the mechanisms of action are not fully elucidated [103]. These observations taken together indicate that particles might interact with the comet assay.

6 DNA REPAIR AND THE MUTAGENIC PROPERTIES OF DNA DAMAGE

DNA lesions can be used as biomarkers of the biologically effective dose of, for example, environmental mutagens or for identification and assessment of different hazardous or protective agents. However, in order to say that DNA damage is a biomarker of cancer risk, this has to be shown in prospective studies, and so far this has not been done for the biomarkers measured with the comet assay. But we know that when DNA damage is left unrepaired, it can lead to mutations, and mutations that are located in the wrong place in the genome can contribute to cancer. DNA repair also plays a crucial role in the long and windy path from DNA lesions to cancer. The relationship between DNA damage and DNA repair is complex and much remains to be elucidated concerning both how this relationship is influenced by gene-environment interactions and by interactions by endogenous factors. Environmental and occupational exposure to chemicals can potentially not only affect DNA lesions but also DNA repair [58]. The first section in this chapter highlights the gradual development of cancer and the many protective pathways that need to fail in order for the disease to develop. In the second section of this chapter, the mutagenic potential of DNA damage (mainly 8-oxoguanine) is discussed. The following sections deal with DNA repair with the focus on the repair of 8-oxoguanine and an application of comet assay; the *in vitro* comet BER assay, which is a new promising tool to measure DNA repair.

6.1 OXIDATIVE STRESS AND CANCER

Oxidative stress is associated with both inflammation and cancer. Inflammation is a response to harmful or injurious stimuli, be it from foreign or endogenous sources, and involves the vascular, as well as immune system. Macrophages formed during inflammation are capable, under the right conditions, of releasing more than hundred substances, including ROS, into the tissue environment to fend off or protect against invading pathogens [104]. The consequences of the inflammatory process, such as the formation of ROS, can also lead to damage to the surrounding cells because reactive compounds are indiscriminating. Some of these damages may occur in such a way that they alter cellular function of surviving cells and with time, additional damage may accumulate and lead to development of disease.

Cancer develops gradually under a long period of time and at least six major pathways protecting the cell must be altered for a cancerous tumour cell to form. These include **a)** self-sufficiency in growth signals, **b)** insensitivity to anti-growth signals, **c)** invasion of apoptosis, **d)** tissue invasion and metastasis, **e)** sustained

angiogenesis, and **f)** limitless replicative potential [105]. Development of cancer is very complex and one must remember that although genetic alterations are important, there are many pieces in the puzzle.

6.2 MUTAGENIC PROPERTIES OF DNA DAMAGE

Chromosome aberrations and micronucleus formation are considered to be biomarkers of early biological effects, reflecting a later step of carcinogenesis compared to biomarkers of exposure [8]. Chromosomal aberrations and micronucleus frequency have been shown to predict cancer risk in prospective studies [106-108]. Whether DNA lesions measured by the comet assay predict cancer risk in prospective cohort studies in a similar manner remains to be evaluated [8]. The biomarkers measured by the comet assay are assumed to provide information about the biologically effective dose [8], and it has been shown that comet assay is effective in identifying carcinogens from non-carcinogens [24, 25]. The mutagenic properties differ substantially between different types of DNA lesions.

6.2.1 The pro-mutagenic potential of 8-oxoguanine

Guanine has a lower redox potential than the other three bases, which makes it particularly susceptible to oxidation [109]. Some of the most common base oxidation products are shown in figure 4. A radical attack at the 8'-carbon of guanine gives the oxidation product 8-oxoguanine (figure 10), which is the most commonly measured oxidative DNA base lesion and an established biomarker for oxidative stress [110, 111].

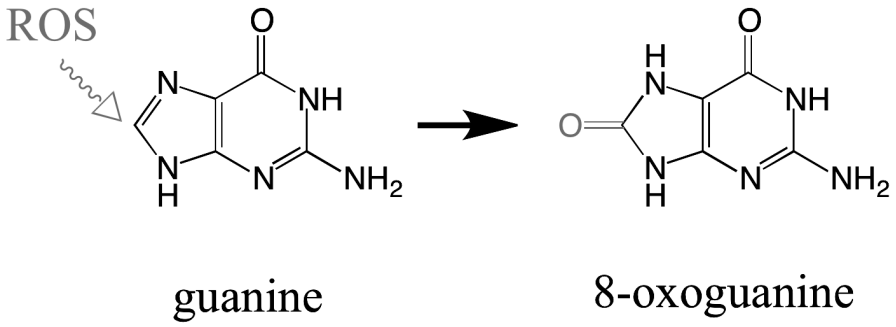

Figure 10. Chemical structures of guanine and the oxidation product 8-oxoguanine.

The extensive interest in 8-oxoguanine by the scientific community is related to both its abundance and its pro-mutagenic potential. Whereas the replication is stalled or inhibited at many other DNA base lesions by the polymerase error detection mechanisms, 8-oxoguanine is pro-mutagenic and can induce a G-C to T-A transversion during DNA replication [112, 113]. 8-oxoguanine pairs with cytosine in the regular *anti* conformation, but can in its *syn* conformation form a stable base pair with adenine [45, 112]. X-ray crystal structures of a DNA polymerase I fragment from *Bacillus stearothermophilus* indicate that adenine is preferred to cytosine opposite 8-oxoguanine during replication, since pairing with cytosine disrupts the active site of DNA polymerase in a similar manner as recognition of a mismatch in undamaged DNA and thereby inhibit further DNA synthesis [112]. With other words, incorrect mutagenic pairing of 8-oxoguanine with adenine can fool the polymerase error detection mechanisms to be treated as a normal base pair [112]. Although most DNA polymerases misincorporate adenine opposite 8-oxoguanine at relatively high frequencies, there are exceptions. Kirouac & Ling recently showed that the specialised human Y-family DNA polymerase iota (polι) does not [114]. The *syn* conformation of 8-oxoguanine does not form a stable base pair with adenine in the narrow active sites of polι, and the correct cytosine is incorporated opposite 8-oxoguanine with a high specificity, albeit with a reduced incorporation efficiency compared to opposite undamaged guanine [114]. Fortunately, organisms have developed a second line of defence against 8-oxoguanines that are mispaired with adenines, where the latter can be removed followed by subsequent removal of 8-oxoguanine (as described in section 6.4). As a result of the efficient repair of 8-oxoguanine, the mutation frequency of 8-oxoguanine in mammalian cells are relatively low (a few percent).

This thesis focuses on 8-oxoguanine since it is the main substrate of Fpg that have been used in papers II-V. However, 8-oxoguanine is not the only DNA lesion that has pro-mutagenic properties. For example, RNS can cause deamination of bases. When hypoxanthine is formed from adenine, the damaged base can mispair with cytosine instead of with thymine as intended. Uracil, which is formed by deamination of cytosine can pair with adenine instead of guanine [3]. Other oxidative DNA lesions with mutagenic potential include for example 5-hydroxycytosine and 5-hydroxyuracil [47]. The two hydantoin products, spiroiminodihydantoin and 5-guanidinohydantoin, are other oxidative modifications of guanine shown to be pro-mutagenic [45]. The mutagenic properties of different DNA lesions is widely assumed to depend on DNA repair since DNA damage that is repaired does not lead to mutations.

6.3 DNA REPAIR

Simplistically, one can say that most oxidative lesions, as well as strand breaks, are repaired by BER, bulky lesions are repaired by NER, replication errors (mismatches) are repaired by mismatch repair, and double strand breaks are repaired by homologous recombination or non-homologous end-joining. It is not the aim of the author of this thesis to review these repair pathways. The focus in this section is on the repair of 8-oxoguanine.

6.3.1 Base excision repair (BER)

Human cells' efficiency in repairing DNA damage as well as the type of repair mechanism that is mainly used depends on the type of DNA damage. The BER is the major pathway for removal of damaged bases, which is performed by several enzymes in cooperation. In the general pathway for short-patch BER, the damaged base is removed by a glycosylase causing an abasic site followed by cleavage of the DNA backbone by either AP-endonuclease, or by an AP-lyase activity that is attached to the glycosylase. Following this, polymerase β, which also contains an associated lyase activity, inserts the correct nucleotide into the DNA strand. The remaining nick on DNA is sealed by ligase III. There are approximately 10 different mammalian glycosylases, of which each is specific for a narrow range of lesions. Most, but not all, glycosylases have associated lyase activities, cleaving the DNA backbone between the phosphate and the sugar (at the 3'-carbon).

6.3.2 Nucleotide excision repair (NER).

Bulky DNA adducts and helix distortions are removed by NER. In NER, an oligonucleotide including the damaged base is removed by a multi-subunit exconuclease complex, followed by synthesis of new bases by DNA polymerase and sealing of the DNA strand by ligase [3].

6.4 REPAIR OF 8-OXOGUANINE

ESCODD estimated the background level of 8-oxoguanine in humans to approximately 0.3-4.2 per million undamaged guanines. This poses a challenge for the enzymes that detect and remove this lesion. The mechanisms of action of these different enzymes are described shortly in section 6.4.1. hOgg1 is considered to be the main enzyme responsible for the removal of 8-oxoguanine in humans. hOgg1 removes 8-oxoguanine when it is paired with cytosine (figure 11). But even after replication has occurred, and 8-oxoguanine has mispaired with adenine, the enzyme MUTYH (previously hMYH) can remove the wrongfully incorporated base (figure 11) [114]. Removal of the mispaired

adenine allows replacement with cytosine by a specialised polymerase, subsequently giving hOgg1 a new chance to repair 8-oxoguanine (figure 11) [114]. MutY, the bacterial parallel to MUTYH, has also been reported to remove adenine misincorporated opposite fapy-guanine, guanine and cytosine [45]. In addition, the NER pathway can probably work as an alternative back-up pathway for removal of 8-oxoguanine [115]. This has been observed in Ogg1 knock-out mice where, in the absence of Ogg1, NER functions as a back-up mechanism for removal of 8-oxoguanine [45]. 8-oxoguanine has also been shown to be removed by the human glycosylase NEIL1, although 8-oxoguanine is not its main substrate [116]. In contrast to Ogg1, which works on double stranded DNA, NEIL1 seems to be particularly active on single stranded DNA and unpaired sequences in bubble DNA, i.e. on intermediates during replication and transcription [116]. Another enzyme often mentioned in this context is MTH1, which degrades 8-oxodGTP from the nucleotide pool by hydrolysing it to 8-oxodGMP, thereby preventing possible misincorporation of damaged guanines into DNA [45]. The bacterial parallels to hOgg1, MTH1 and MUTYH are called Fpg/MutM, MutT and MutY.

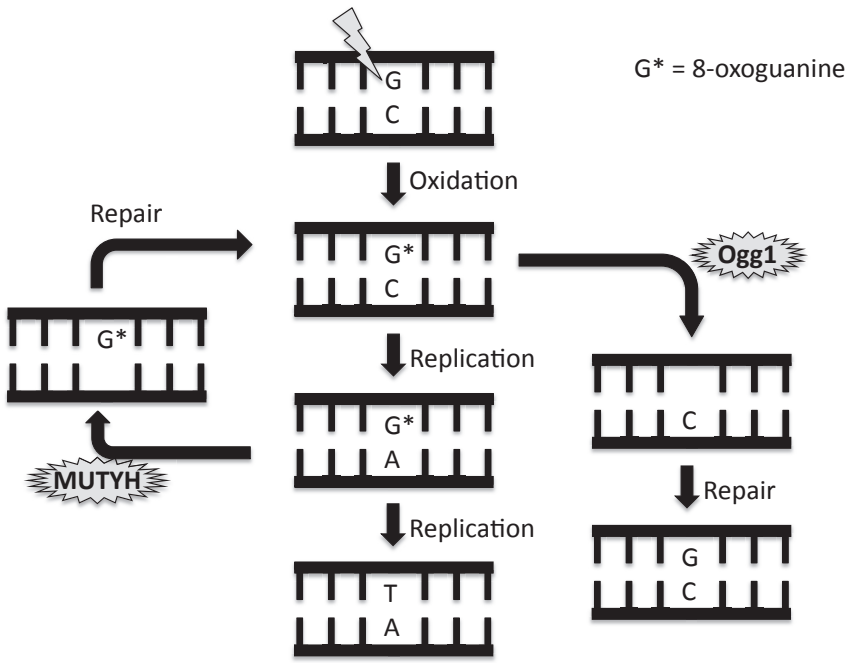

Figure 11. Schematic description of the path from oxidation of guanine to the completed G-C to T-A transversion (illustrated by the pathway in the middle), and how the repair enzymes Ogg1 and MUTYH can prevent this (illustrated by the right and left pathways, respectively).

6.4.1 Glycosylases' mechanism of action

The glycosylase slides along the DNA chain. Frequently, it forms an 'interrogation' structure where bases flip out from the DNA helix and are inspected by the enzyme extra-helically. When encountering a damaged base, it is transferred from the interrogation complex to the enzyme's active site [39]. Bifunctional glycosylases, such as Ogg1 and Fpg, possess both glycosylase and AP-lyase activity, cleaving first the glycosidic bond between the base and the sugar and then the DNA backbone (at the 3'-carbon of the abasic sugar) [40, 46]. Fpg also have an attached AP-endonuclease activity cleaving the other phosphodiester bond at the DNA backbone (at the 5'-carbon of the abasic sugar) [46].

6.4.2 The active site of hOgg1

The BER process of 8-oxoguanine in humans is a multistep process, which is considered to be very lesion-specific. 8-oxoguanine is deeply inserted into the specificity pocket of hOgg1, and there is a range of interactions between the enzyme and 8-oxoguanine, with the phosphate moieties on the damaged DNA strand and the cytosine moiety on the complementary strand [49]. David *et al.* highlights two interactions between Ogg1's active site and 8-oxoguanine as particularly important for the recognition of 8-oxoguanine and rejection of guanine; a dipole-dipole interaction enabled by the local dipole opposite direction compared to guanine (as a result of the charge inversion at the 7'-nitrogen and the 8'-carbon), and a 8-oxoguanine-specific hydrogen bond between the hydrogen at the 7'-nitrogen and a glycin residue in Ogg1's active site (Gly 42) [45].

In contrast, Fpg, the bacterial parallel to hOgg1, has been shown to recognise a range of different DNA-lesions (see section 3.2.4). In spite of these two enzymes' similar roles, they are not structurally related to each other [39]. In fact, Ogg1's structure is quite similar to that of AlkA [49].

6.5 MEASURING DNA REPAIR

The DNA repair has often been neglected in human biomonitoring studies, which in part is explained by the limitations of the available methods [52]. Some of the drawbacks of 'the challenge assays' (where damage is induced and allowed to be repaired) are that they are laborious, demanding for example cell culturing or multiple sampling and that data might be difficult to interpret [52]. It is not uncommon to measure expression of DNA repair genes, although mRNA levels often are found not to correlate with enzyme activity. Paz-Elizur *et al.* argue that an advantage of measuring enzymatic repair activity is that it includes the influence of genetic polymorphism, epigenetic effects,

transcription factors, splicing, RNA stability, translation factors, protein stability and possibly environmental and life style factors [117]. Compared to the frequently measured mRNA levels or SNP analyses, it can be argued that assays estimating real DNA repair activity might provide more substantial information. DNA repair capacity can be assessed by extracting DNA repair enzymes from cells and measure the extract's ability to repair DNA lesions. The efficiency of both BER and NER can be measured by modified versions of the comet assay [17-20], and the agent used to induce the damage defines the specificity of the assay [52]. These relatively new applications of the comet assay are likely to receive growing attention in the future. Much remains to be done in the validation of these *in vitro* comet BER and NER assays. We have performed a validation study of the *in vitro* comet BER assay within ECVAG (study 3, section 4.2), where the reliability and reproducibility of the assay was investigated (Godschalk *et al*., in preparation). The preliminary findings indicate that six of eight laboratories could detect DNA repair incisions and that there was some consistency in the ranking of these samples although the reported levels varied substantially (Godschalk *et al*., in preparation). As with the comet assay, there is no standardised protocol for the *in vitro* comet BER assay. For example, different researchers **a)** use 10 or 20 min incubation with the extract containing repair enzymes, **b)** report outcome in different ways, and **c)** use different comet assay protocols. In addition, when an up-regulation of repair is observed, it is recommended to make sure that this is not in fact a direct damaging effect of the investigated compound or an induction of non-specific nucleases [118].

7 ANTIOXIDANTS

An antioxidant is any substance, which delays or inhibits oxidative damage to a target molecule. Some antioxidants are supplied by the diet and others are endogenously synthesised, consisting of both enzymatic and non-enzymatic antioxidants. Antioxidants defend the body against oxidative injury and are widely assumed to provide a protection against various diseases. The diet has been estimated to account for 30-35% of the overall cancer risk. A balanced diet, rich in fruit and vegetables, contains a mixture of antioxidants, and is related to a reduction of cardiovascular diseases and cancer incidence [119, 120].

Recent reports from the national health and nutrition examination survey (NHANES) document that 49% of the U.S. men, women and children and 54% of the adults included in the study between 2003-2006 consumed dietary supplements [121]. Multivitamin supplements (≥3 vitamins, with or without minerals) were consumed by 40% of the adults in the study [121]. The use of dietary supplements has increased substantially over time since the first NHANES study in 1971-1974 [121, 122]. Supplements worth more than $20 billion/year are sold in the U.S. alone [123].

Studies investigating the beneficial effects of antioxidant supplements, often containing a single or a few antioxidants in relatively high doses, have been inconclusive. The protective effects of antioxidant supplements containing for example β-carotene, vitamin A and vitamin E have been questioned [124]. These results are likely to be explained, at least in part, by the fact that the protective effect of antioxidants is a combinatory effect of numerous antioxidants co-operating and that the delicate balance between antioxidants in the body might be disturbed by supplementing one or a few antioxidants in high doses. Antioxidants might even under certain conditions have pro-oxidant effects [125], and antioxidants supplied by a balanced diet are preferred to dietary supplements, which contain a few antioxidants in high doses [119]. However, whereas antioxidant supplementation to a mal-nutritious population could be of benefit, administration of supplements with antioxidants to individuals who are well nourished might lead to misleading conclusions.

7.1 LARGE RANDOMISED CLINICAL INTERVENTION STUDIES WITH ANTIOXIDANTS

In this section, a selection of the largest, randomised, placebo-controlled, double-blind antioxidant intervention studies with good design will be described and discussed. These include **a)** the Linxian general population trial, **b)** the α-tocopherol, β-carotene cancer prevention study (ATBC), **c)** the β-carotene and retinol efficacy trial (CARET),

d) the physicians' health study, **e)** the supplémentation en vitamines et minéraux antioxydants (SU.VI.MAX) study, **f)** the women's health initiative, and **g)** the selenium and vitamin E cancer prevention trial (SELECT). Three studies (studies **b**, **d** and **g**) had a 2^2 factorial design meaning that the subjects received one out of two antioxidants, both antioxidants, or placebo. Study **a** had a 2^4 factorial design, and in studies **c**, **e** and **f** the participants consumed either a multivitamin supplement or placebo.

7.1.1 The Linxian general population trial

The Linxian general population trial, which started in 1986 included 29,584 Chinese men and women aged 40-69 with a poor overall nutritional status, who consumed supplements with different combinations of vitamins and minerals or placebo daily for 5.25 years [126-128]. Significant reductions of total mortality (9%), total cancer mortality (13%) and stomach cancer mortality (21%) was observed in the participants that had received 30 mg α-tocopherol, 15 mg β-carotene and 50 μg selenium (as selenium yeast) [126]. Significant beneficial effects of this treatment could still be seen in a 10-year follow up study, with a 5% reduction of total mortality (almost entirely due to effects in subjects younger than 55 years) and a 11% reduction of gastric cancer mortality [128]. The other three combinations of antioxidants did not affect mortality [126].

7.1.2 The ATBC study

The ATBC study, which was initiated in 1985 included 29,133 Finish male smokers aged 50-69 years who consumed 20 mg β-carotene and/or 50 mg α-tocopherol or placebo daily for 5-8 years (median 6.1 years) [129-131]. Significant increases of total mortality (8%), lung cancer incidence (16%) and non-significant increases of prostate cancer (23%) and stomach cancer (25%) were observed in the subjects who consumed β-carotene supplements [129, 131, 132]. A significant 34% reduction of prostate cancer, a non-significant decrease of colorectal cancer (16%) and a non-significant increase of stomach cancer (25%) were observed in the subjects who consumed α-tocopherol supplements [129]. Although the harm of the β-carotene supplement was not as evident a few years after the study had been terminated, a non-significant 17% increase of lung cancer incidence was seen during a 3-year follow up study, and a 7% significantly higher mortality was seen during a 8-year follow up study of the subjects that had received β-carotene [131].

7.1.3 The CARET trial

The CARET trial, which was initiated in 1983 (and was expanded in 1988 and 1991) included in total 18,314 U.S. male and female heavy smokers and former-smokers and/or male workers occupationally exposed to asbestos, aged 45-74, who consumed

30 mg β-carotene and 25,000 IU retinyl palmitate (vitamin A) or placebo daily for an average of 4 years [133-135]. A significantly increased lung cancer risk of 28% was seen in supplemented subjects, including a 42% increased risk for smokers and a 40% increased risk for workers exposed to asbestos [135]. In addition, a significantly increased all-cause-mortality of 17%, and a significantly increased lung cancer mortality of 46% was observed in the subjects who consumed supplements [135]. After considering the outcome of the ATBC study and evaluating the effect of supplementation on mortality and cancer incidence, the CARET trial was terminated in January 1996, 21 months ahead of the planned end [133, 135]. A harmful non-significant effect of treatment remained during a 6-year follow up study, including non-significant increases of all-cause mortality (8%), lung cancer mortality (20%) and lung cancer incidence (12%), with a more persistent effect seen in females than in males [133].

7.1.4 The physicians' health study I

The physicians' health study I, which was initiated in 1982, included 22,071 U.S. male physicians aged 40-84 who consumed 50 mg β-carotene every second day for 11.6-14.2 years (average 12 years), and/or 325 mg aspirin every second day (although this administration was terminated already after 5 years) or placebo [136]. Supplementation did not significantly influence any of the assessed parameters including all-cause mortality, cancer mortality and lung cancer incidence [136]. No effect of β-carotene supplementation on lung cancer was seen among the 11% smokers or 39% former smokers in this study [136].

7.1.5 The SU.VI.MAX study

The SU.VI.MAX study, which was initiated in 1994 consisted of 13,017 French men and women aged 35-60 years who consumed either a multivitamin and mineral supplement consisting of 120 mg Vitamin C, 30 mg α-tocopherol, 6 mg β-carotene, 100 μg selenium (as selenium yeast) and 20 mg zinc or placebo daily during 7.5 years (median) [137]. The multivitamin supplement significantly decreased the risk of developing all types of cancer by 31% and all-cause mortality by 37% in men, but not in women [137]. Among the men with a normal baseline level of prostate-specific antigen (PSA) that were supplemented with β-carotene, the incidence of prostate cancer was significantly decreased with 48% [138].

7.1.6 The women's health initiative

The women's health initiative was a 15-year research program initiated in 1991 that included 161,808 postmenopausal women and consisted of an observational study and several clinical trials. One of these trials included 36,282 U.S. postmenopausal women

aged 50-79 years who consumed 1 g of calcium (as calcium carbonate) and/or 400 IU of Vitamin D_3 or placebo daily during 7 years (on average) [139]. Supplementation did not significantly affect the incidence of colorectal cancer, all types of cancer, all-cause mortality, total cancer mortality or colorectal cancer mortality [139].

7.1.7 The SELECT trial

The SELECT trial, which was initiated in 2001 consisted of 35,533 men from U.S., Canada and Puerto Rico aged >50 years who consumed 200 μg selenium (from *L*-selenomethionine) and/or 400 IU α-tocopherol or placebo each day for 4.2-7.3 years (median 5.5 years) [140]. The antioxidant supplement did not affect the incidence of prostate cancer, lung cancer, colorectal cancer, all types of cancer or all-cause mortality after 7 years, and therefore, the study, which was planned to continue for 12 years was terminated after 7 years [140].

7.2 WHAT CAN WE LEARN FROM THESE STUDIES?

Taken together, these large randomised clinical trials have not provided proof of a beneficial effect of antioxidant supplements. Instead, they raise questions regarding **a)** lifestyle, **b)** harm and benefit to different subgroups, **c)** appropriate doses, and **d)** types, forms and combinations of antioxidants.

7.2.1 Smokers

Results from the ATBC and CARET trials indicate that β-carotene supplementation increases smokers' lung cancer risk [130, 134]. However, it shall be emphasised that the participants in these studies consisted of heavy smokers that cannot be regarded to represent a healthy population. β-carotene supplementation were particularly harmful to the subjects who drank more and who smoked the most [132, 134]. The participants in the ATBC study smoked on average 20.4 cigarettes per day and had smoked for an average of 35.9 years [130]. Participants supplemented with β-carotene who smoked more than 20 cigarettes/day had a significantly 25% increased risk of developing lung cancer, whereas those who smoked between 5-19 cigarettes/day had a non-significantly 3% decreased risk of developing lung cancer [132]. In the CARET trial, a significant 28% increased risk of lung cancer was observed in supplemented subjects. Interestingly, in the subjects not exposed to asbestos, the effect was largest in current smokers, i.e. smokers at baseline, where a 42% increased lung cancer incidence was reported [134]. On the other hand, supplementation did not affect the incidence of lung cancer in the former smokers not exposed to asbestos [134]. The smokers and former smokers in the CARET trial were very heavy smokers or former smokers with at least 20, and an average of 49 pack-years of cigarette smoking [134]. The asbestos-exposed workers had

been occupationally exposed to asbestos for an average of 27 years [134]. In the physicians' health study where 11% were smokers and 39% former smokers, smoking did not significantly increase or decrease the risk of lung cancer [136]. However, the participants in the physicians' health study had a relatively low risk of lung cancer (6.6% of the total cancer incidence) [136, 141].

7.2.2 Poorly nourished individuals

The Linxian study reported significant beneficial effects of supplementation for the participants that had received α-tocopherol, β-carotene and selenium [126]. The participants in the Linxian general population trial is a high-risk population with regards to a very high incidence of oesophageal cancer and a poor overall nutritional status with low intake of several micronutrients, i.e. vitamins A, B_2, C, E, selenium, zinc and calcium [142, 143]. In the SU.VI.MAX study, supplementation with multivitamins significantly decreased the risk of developing all types of cancer and all-cause mortality in men, but not in women [137]. This could be attributed to that the baseline intake of antioxidants was lower in men than in women [137].

7.2.3 Healthy subjects

In supplementation studies with healthy subjects, such as the physicians' health study [136], the women's health initiative [139], the SELECT trial [140], and the women in the SU.VI.MAX study [137], no effects of antioxidant supplements on cancer incidence have been observed.

There is some evidence that antioxidant supplements can be particularly harmful to certain subgroups. Factors that can affect the individual's response to antioxidant supplementation include genetics, lifestyle, deficiency, diseases and pre-existing tumours [144]. At a whole, the large intervention studies described in this chapter suggest that; **a)** supplementation with β-carotene can be harmful to smokers, and the risk seem to differ for former smokers, smokers, and heavy smokers, **b)** subjects with deficient or low intake of antioxidants can be particularly benefited by supplementation, and **c)** neither harmful nor beneficial effects was seen by supplementation to healthy individuals.

7.2.4 Doses

The dose of β-carotene is worth mentioning since the participants' serum concentrations of β-carotene increased 17-fold and 12-fold in the ATBC study and the CARET trial, respectively [129, 135], whereas in the physicians' health study I, the serum concentrations of β-carotene increased only 4-fold [136]. Worth mentioning is also that a

lower dose of β-carotene was administered in the Linxian trial compared to the ATBC and CARET trials [127, 130, 134].

7.2.5 Baseline values

When looking closer at baseline serum levels of antioxidants and antioxidant intake in the studies described above, several interesting relationships with cancer incidence are observed. In the ATBC study, dietary intake and serum concentrations of β-carotene and α-tocopherol at baseline were inversely associated with lung cancer incidence in the control group [129]. In the CARET trial, serum β-carotene concentrations at baseline were inversely associated with lung cancer incidence in both subjects supplemented with antioxidants and with placebo [135]. In addition, consumption of fruit and cruciferae vegetables (e.g. broccoli) at baseline significantly protected against lung cancer in the placebo group (but not in the treatment group) [145]. In SU.VI.MAX's placebo group, the male quintile with the highest serum β-carotene concentrations at baseline had a significantly 45% decreased incidence of cancer compared to the lowest quintile [137]. No such effect was seen for women [137]. The investigators discuss that the significant effect seen in men, but not in women, could be related to that the men had both a relatively low antioxidant intake and lower baseline concentrations of antioxidants compared to women [137]. In the women's health initiative, serum 25-hydroxyvitamin D at baseline was significantly inversely associated with colorectal cancer, but baseline levels did not affect the outcome of the supplementation [139]. In the physicians' health study I, the men with the lowest quartile of plasma β-carotene levels at baseline, had a non-significant 30% increased incidence of cancer compared to those in the highest quartile [141]. The subjects with the lowest quartile of plasma β-carotene levels that were supplemented with β-carotene had a non-significant 17% decreased risk of cancer and a statistically significant 32% decreased risk of prostate cancer (which is the most frequent form of cancer in this group) [141]. In a 15-year follow up study to the Linxian study, consumption of fresh fruit at baseline was shown to significantly protect against oesophageal squamous cell carcinoma and gastric cardia cancer but not against gastric non-cardia cancer [142].

A reason often proposed for the discrepancies between observational studies and clinical intervention studies is that serum levels of certain antioxidants are biomarkers for a healthy diet, rich in fruits and vegetables, and that it is not individual antioxidants that are responsible for the beneficial effects. The antioxidant intake and serum levels at baseline, which can be expected to reflect a healthy diet, clearly are inversely related to cancer incidence in the studies described above. The results from the Linxian study, the physicians' health study and the SU.VI.MAX study support the hypothesis that supplementation can be beneficial for poorly nourished individuals. Although, it is

possible that the dietary intake is a better marker for a long-time consumption than antioxidant supplements, and therefore has a larger impact on cancer, which develops gradually under a long period of time [144]. Whether the beneficial effects are related to a balance between different micronutrients and minerals working together and/or beneficial effects of other food constituents, such as fibres, remains to be fully elucidated.

7.3 ANTIOXIDANT INTERVENTION STUDIES AND THE COMET ASSAY

Biomarkers both of exposure and biological effects are valuable tools when trying to elucidate the complex relationship between exposure and disease. In addition, studies using biomarkers are cheaper, faster and require fewer subjects than large intervention studies with disease and/or mortality as endpoints. An important area where biomarkers may serve as indicative measures, is within nutrition and its impact. This chapter contains a brief overview of the antioxidant intervention studies that have measured oxidative DNA lesions (but not DNA breaks) with the comet assay. A selection of intervention studies with antioxidant supplements is presented in the following section. Møller & Loft have in two review studies reported that antioxidant intervention studies with a weak design is more likely to show a protective effect against oxidative DNA damage [146, 147]. The authors discuss that a randomised parallel placebo-controlled design is the optimal study design [146], and that a frequent problem is that studies lack power to detect small changes, i.e. that too few subjects have been used [146, 147]. Only parallel randomised, placebo-controlled intervention studies with more than 10 subjects, where antioxidants have been administered in multiple doses (ranging for weeks or months) are discussed in the following section. The subsequent section covers biomonitoring studies with nutritional modulation with food items containing fruits and/or vegetables. Only fruits and vegetable-related food items are discussed since these are most relevant to compare with the intervention study in paper IV. However, other food items (for example coffee) have also been shown to have protective effects. When supplementation is performed with food items, placebo-controlled intervention studies are often not feasible, and therefore studies where seasonal changes have been taken into account, i.e. randomised placebo-controlled parallel studies, and controlled studies with either parallel or cross-over design, are discussed. Again, only studies with more than 10 subjects, where the intervention has lasted for weeks/months are discussed. In addition, when assessed, information about DNA repair capacity and protection against DNA breaks induced *ex vivo* with H_2O_2, is provided for these studies in order to give a more complete picture.

7.3.1 Antioxidant supplements

Healthy men and women who received either α- and β-carotene, lutein or lycopene for 12 weeks had no significant differences in Fpg-sensitive sites or EndoIII-sensitive sites after intervention compared to subjects receiving placebo, in spite of significantly increased serum levels of the antioxidants [148]. In another study, significantly decreased levels of EndoIII-sensitive sites was seen after a six weeks intervention with 500 mg rutin or placebo (in both treatment groups), although no significant differences were seen between the supplemented subjects and the subjects receiving placebo [149]. In addition, supplementation did not provide any protection against H_2O_2-induced DNA breaks, nor did it affect urinary levels of MDA or 8-iso-$PGF_{2\alpha}$ [149]. In a 24 day-intervention with a multivitamin supplement roughly corresponding to the content found in 600 g of fruit and vegetables, no significant effect on Fpg-sensitive sites, EndoIII-sensitive sites or protection against H_2O_2-induced DNA breaks was observed in healthy non-smoking men and women [150]. Also, BER capacity measured by the *in vitro* comet BER assay was unaffected by the intervention [151]. However, in a study with poorly nourished smokers with relatively low plasma vitamin C levels and relatively high EndoIII- and Fpg-sensitive sites at baseline, consuming 500 mg slow release vitamin C and 182 mg plain release α-tocopherol during 4 weeks, significantly reduced levels of EndoIII-sensitive sites and Fpg-sensitive sites were seen after compared to before supplementation [151, 152]. However, no significant differences were observed before and after supplementation in subjects receiving plain release vitamin C and α-tocopherol or placebo [152]. When investigating BER capacity using the *in vitro* comet BER assay, a significant increase of BER was observed after supplementation of slow release vitamin C and α-tocopherol for four weeks, whereas no effects by intervention were seen in the plain release vitamin C group or the placebo group [151]. Healthy non-smoking and smoking men who were supplemented with 100 mg vitamin C, 280 mg α-tocopherol and 25 mg β-carotene daily for 20 weeks had significantly elevated plasma levels of all supplemented antioxidants and significantly decreased levels of EndoIII-sensitive sites after supplementation compared to subjects receiving placebo [153].

We have investigated the effect of consumption of antioxidant supplements consisting of extracts from fruit, berries and vegetables, in two randomised parallel double-blind placebo-controlled intervention studies with healthy middle-aged overweight men [154] and type 2 diabetes subjects [155], respectively. The first study, which is included in this thesis (paper IV), is described thoroughly in chapter 8 [154]. In the second study, subjects with a high intake of fruits and vegetables and relatively well-controlled type 2 diabetes consumed antioxidant supplements or placebo daily for 12 weeks [155]. Antioxidant supplementation did not affect the level of any of the measured biomarkers of oxidative stress, i.e. 8-iso-$PGF_{2\alpha}$ in urine, plasma MDA, 8-oxodG/dG, Fpg-sensitive sites, plasma

nitrotyrosine or micronuclei (frequency of micronucleated transferring-positive reticulocytes), or inflammation, i.e. high sensitivity C-reactive protein (hs-CRP), interleukin 6 (IL-6) or 15-keto-dihydro-prostaglandin $F_{2\alpha}$, in spite of highly significantly ($P < 0.001$) elevated levels of serum/plasma levels of six out of seven measured antioxidants (γ-tocopherol, vitamin C, α-carotene, β-carotene, lycopene and lutein) [155].

7.3.2 Nutritional modulation

In a randomised placebo-controlled cross-over study, male smokers were supplemented with a vegetable burger (equivalent to ~500 g mixed fresh vegetables) and a fruit juice (consisting of juice concentrates of orange, blueberry, apple, lemon and lime) for three weeks, with a wash-out phase of two weeks between the treatment and placebo [156]. In spite of highly significantly elevated serum levels of vitamin C and several carotenoids (α-carotene, β-carotene, β-cryptoxanthin and zeaxanthin), the antioxidant treatment did not affect the level of EndoIII-sensitive sites or the level of lipid peroxidation (MDA and 8-epi-prostaglandin $F_{2\alpha}$), nor did it protect against H_2O_2-induced DNA breaks [156]. Similarly, in a parallel dietary randomised placebo-controlled intervention study where healthy men and women consumed 600 g of fruits and vegetables, or a placebo tablet for 24 days, the supplementation did not significantly alter the level of Fpg-sensitive sites or EndoIII-sensitive sites, nor did it protect against H_2O_2-induced DNA breaks [150]. In a subsequent study of BER capacity using the *in vitro* comet BER assay (on the same subjects), no effect was observed by intervention [151]. On the other hand, in a randomised study with cross-over design where healthy men and women consumed 1, 2 or 3 green kiwifruits daily during three weeks supplementation periods, with two weeks wash-out periods between treatments, significantly lower levels of EndoIII- and Fpg-sensitive sites were observed after three weeks [157]. Kiwifruit consumption induced an increased BER capacity as measured by the *in vitro* comet BER assay, and significantly protected against H_2O_2-induced DNA breaks [157]. In a recent similar randomised cross-over study, healthy men and women eating modest amount of fruits and vegetables consumed 1 or 2 golden kiwifruits during four weeks with four weeks wash-out periods between supplementations [158]. Significantly decreased levels of both EndoIII- (after two kiwifruits/day) and Fpg-sensitive sites (after one kiwifruit/day) were observed after four weeks of supplementation [158]. Kiwifruit consumption significantly protected against H_2O_2-induced DNA breaks, but neither BER nor NER were altered by supplementation as measured by the *in vitro* comet BER and NER assays [158]. In a randomised parallel controlled intervention study healthy men and women drank 475-1000 ml (based on body weight) of blackcurrant juice (rich in anthocyanins and vitamin C), an anthocyanin drink, or a placebo drink daily during three weeks [159]. No statistically significant effect on either EndoIII-sensitive sites or

Fpg-sensitive sites was observed between the supplemented subjects and the subjects consuming placebo [159]. There was, however, an increased level of Fpg-sensitive sites after compared to before intervention in the subjects who consumed blackcurrant juice [159]. In a similar randomised parallel placebo-controlled intervention study where healthy women drank 750 ml cranberry juice or a placebo drink daily for two weeks, no effect was observed on EndoIII-sensitive sites nor was there any protection against H_2O_2-induced DNA breaks, in spite of a significant increase of vitamin C in plasma in the subjects drinking cranberry juice [160].

In a controlled randomised cross-over study where male smokers and non-smokers consumed 200 g steamed broccoli or a control diet daily for 10 days with a 20 day wash-out period between the diets, the level of Fpg-sensitive sites decreased significantly after supplementation with broccoli, but when dividing the subjects into smokers and non-smokers, the decrease in Fpg-sensitive sites was only significant in the smokers [161]. In addition, the broccoli supplementation significantly protected against H_2O_2-induced DNA breaks [161]. In a similar randomised cross-over study, male smokers consumed 250 g steamed broccoli or a control diet daily for 10 days with a 20 day wash-out period between the diets [162]. A significant protection against H_2O_2-induced DNA breaks and significantly decreased levels of Fpg-sensitive sites was seen after consumption of broccoli but not after consumption of the control diet [162]. BER capacity as measured by the *in vitro* comet BER assay was not altered by the intervention [162]. In a recent large controlled randomised parallel intervention study (n = 102), healthy male smokers consumed either **a)** a diet rich in antioxidants, **b)** three kiwifruits, or **c)** a controlled normal diet, daily during eight weeks [163]. None of the interventions affected the levels of EndoIII- or Fpg-sensitive sites, nor did they protect against H_2O_2-induced DNA breaks [163]. A significantly increased BER capacity, and a significantly decreased NER capacity was observed after intervention in the subjects who consumed a diet rich in antioxidants as measured by the *in vitro* BER and NER comet assays [163]. A significant decreased NER capacity, and a non-significant increased BER capacity was observed following supplementation with kiwifruit [163].

8 THE INTERVENTION STUDY WITH OVERWEIGHT MIDDLE-AGED MEN

In a clinical placebo-controlled double-blind parallel intervention study, 60 overweight or obese (BMI 25-40) men, aged 36-66 years, were supplemented with a multivitamin-multimineral supplement or placebo during six weeks (papers IV-V) [164]. The subjects were randomly divided into a placebo group (n = 20), a single-dose group (n = 21) and a double-dose group (n = 19). Biomarkers of oxidative stress, metabolic variables and antioxidant levels in serum or plasma were assessed before and after treatment [154]. The intervention study with all measured parameters are described in paper IV [154], whereas paper V is based on a potentially interesting observation from the intervention study that has also been further investigated in an *in vitro* cell study [164].

8.1 THE ANTIOXIDANT SUPPLEMENT

It was hypothesised that a range of antioxidants in doses resembling those achieved by a healthy diet would be more beneficial to health than the high doses of one or a few antioxidants that are frequently used in intervention studies with antioxidants [154]. An antioxidant supplement, which consisted mainly of a combination of extracts derived from fruits, berries and vegetables, was developed by Dr. E. Rytter. The antioxidant contents of the supplements administered to the two dose groups are shown in Table 1. The content of antioxidants in the supplements described in paper V (Table II) was incorrect, and should have been multiplied by a factor 2 [164]. The antioxidant content in the supplements administered to the single-dose group approximately corresponded to the intake recommended by the Swedish national food administration (500 g of fruits, berries and vegetables).

Table 1. Characterisation of the antioxidant content administered to the two dose groups in the intervention study in with overweight middle-aged men (paper IV).

Ingredient	Antioxidant	Content	
		Single dose group	Double dose group
Green tea extract	Catechins	160 mg	320 mg
Bilberry extract	Anthocyanidins	6 mg	12 mg
Rosehip extract	Ascorbic acid	90 mg	180 mg
Acerola extract	Ascorbic acid	30 mg	60 mg
Citrus extract	Flavanones + Flavones	40 mg	80 mg
Grape seed extract	Proanthocyanidins	48 mg	96 mg
Cranberry extract	Quinic acid	22 mg	44 mg
Rutin	Rutin	60 mg	120 mg
Vegetable oil concentrate	α-tocopherol	20 mg	40 mg
	β-tocopherol	1.44 mg	2.88 mg
	γ-tocopherol	88 mg	176 mg
	δ-tocopherol	32 mg	64 mg
Carrot extract	α-carotene	4 mg	8 mg
	β-carotene	8 mg	16 mg
Tomato extract	Lycopene	3 mg	6 mg
Marigold extract	Lutein	4 mg	8 mg
Garlic oil	not specified	–	–
Thyme oil	Thymol	120 µg	240 µg
	Carvacrol	8 µg	16 µg
Artichoke extract	Cynarin	3 mg	6 mg
Selenium chelate and yeast	Selenium	100 µg	200 µg
Zinc sulphate	Zinc	14 mg	28 mg

8.2 METHODS USED IN PAPER IV

Several biomarkers for oxidative stress were measured both before and after supplementation of antioxidants or placebo. As biomarkers of oxidatively damaged DNA, Fpg-sensitive sites were measured by the comet assay [164] and the level of 8-oxodG/10^6 dG was measured by HPLC-EC/UV [165]. As biomarkers of lipid peroxidation, 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was measured by a radioimmunoassay [166] and malondialdehyde (MDA) was measured by HPLC with fluorescence detection [167]. In addition, plasma or serum levels of eight different antioxidants as well as cholesterol and triacylglycerol concentrations were measured before and after antioxidant supplementation.

8.2.1 Analysis of 8-oxodG with HPLC/EC/UV

8-oxodG/10^6 dG was measured by the HPLC-EC/UV method as described by Hofer & Möller [165]. On-line EC detection after separation with HPLC is a common method for analysis of 8-oxodG. 8-oxodG is oxidised in the electrochemical cell, which gives rise to a current that is proportional to the concentration of 8-oxodG. Non-damaged dG is measured by UV detection. The ratio between 8-oxodG and dG gives an estimate of the

level of oxidative stress. Artefact oxidation during the workup procedure of DNA has been shown to be responsible for overestimated levels of 8-oxodG (discussed in section 4.1). An extensive effort to optimise and validate the 8-oxodG HPLC-EC/UV detection method has previously been done by Lennart Möller's research group [62, 64, 168]. In order to reduce oxidation during sample preparations, chilled centrifuges as well as high purity chemicals and enzymes were used, and workup was performed on ice. In addition, the antioxidant enzyme catalase from *Aspergillus Niger* and the electron acceptor 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO, a free radical) were used, and solutions were treated with chelex 100 resin to remove catalytic redox cycling transition metals [165, 168].

8.2.2 Analysis of Fpg-sensitive sites with the comet assay

Fpg-sensitive sites were measured by a low-alkaline version of the comet assay that is thoroughly described in paper V [164]. The method is substantially different from the method employed by the author of this thesis (described in detail in paper III) [72]. When comparing the two protocols, the method used in the intervention study with overweight middle-aged men is very time consuming, including a 1h wash after lysis, a 1h incubation with enzyme buffer, a 30 min incubation with enzyme for penetration (cold), a 1 h incubation with enzyme at 37°C and two 45 min incubations in different alkaline buffers before electrophoresis. In addition, the units and composition of the lysis and alkaline solutions, differ and the alkaline unwinding and electrophoresis were performed at room temperature.

8.2.3 Analysis of MDA

Plasma MDA levels were measured by HPLC with fluorescence detection as described by Öhrvall *et al.* [167]. MDA is the most commonly measured biomarker of lipid peroxidation. MDA is frequently incubated with thiobarbituric acid (TBA), with which it forms a fluorescing complex that can be measured. However, TBA binds to other substances as well, and the reaction is regarded to be unspecific. In order to minimise interference by other substances that bind to TBA, MDA-TBA adducts were separated from contaminants by HPLC and subsequently measured with fluorescence detection [167]. Although the reported levels of MDA were not exceedingly high, artefact oxidation during incubation of TBA with plasma cannot be excluded, since the incubation was performed at a high temperature (~100°C) [169]. A range of more specific methods to measure MDA levels have been developed since the intervention study in paper IV took place (in 2000) [169].

8.2.4 Analysis of 8-iso-$PGF_{2\alpha}$ by a radioimmunoassay

Another biomarker of lipid peroxidation, 8-iso-$PGF_{2\alpha}$ in urine, was measured with a radioimmunoassay as described by Basu [166]. 8-iso-$PGF_{2\alpha}$, is a prostaglandin derivative synthesised through free radical catalysed peroxidation of arachidonic acid, a polyunsaturated omega-6 fatty acid. In short, a known amount of 8-iso-$PGF_{2\alpha}$ was made radioactive by labelling with tritium and the radioactive 8-iso-$PGF_{2\alpha}$ was then mixed with an antibody specific for 8-iso-$PGF_{2\alpha}$ (although it also possess certain cross-reactivity to several compounds that is structurally related to 8-iso-$PGF_{2\alpha}$) [166]. When the sample is added to the mixture, the 8-iso-$PGF_{2\alpha}$ from the sample competes with the radioactive 8-iso-$PGF_{2\alpha}$ bound to the antibodies, leading to release of 'free' radioactive 8-iso-$PGF_{2\alpha}$. By separation of the bound 8-iso-$PGF_{2\alpha}$ from the radioactive 'free' 8-iso-$PGF_{2\alpha}$, the latter could be determined using a β-liquid scintillation counter [166]. The level of variation of 8-iso-$PGF_{2\alpha}$ in urine was corrected for by adjustment to the urinary creatinine concentration [154].

It can be argued that 8-iso-$PGF_{2\alpha}$ in urine is a more suitable biomarker than 8-iso-$PGF_{2\alpha}$ in plasma when assessing the effect of a dietary intervention (when expecting decreased levels), since the substance is more abundant in urine than in plasma [166].

8.2.5 Analysis of antioxidants and metabolic variables

Measurements of serum α- and γ-tocopherol, serum carotenoids (α-carotene, β-carotene, lycopene and lutein), plasma selenium, plasma selenoprotein P, serum cholesterol and triacylglycerol concentrations were analysed by experienced investigators as described in paper IV [154].

8.3 FINDINGS

Highly significantly ($P < 0.001$) elevated levels of serum γ-tocopherol, serum α-carotene, serum β-carotene, serum lycopene, serum lutein, plasma selenium and plasma selenoprotein P were observed in both dose groups compared to the placebo group after six weeks of intervention [154]. In addition, the subjects that received the double-dose had significantly higher levels of serum γ-tocopherol, serum α-carotene, serum β-carotene, serum lycopene and plasma selenoprotein P, and non-significantly higher levels of plasma selenium and serum lutein, compared to subjects receiving the single-dose [154]. The serum α-tocopherol concentration was not significantly altered by supplementation. In spite of the elevated levels of seven out of eight measured antioxidants, the intervention did not alter any of the four measured biomarkers for oxidative stress. In addition, no effect was seen on serum triacylglycerol, serum

cholesterol or BMI in any of the treatment groups after six weeks of antioxidant supplementation [154].

Although the decrease of Fpg-sensitive sites in the double-dose group was not significant after compared to before intervention [154], the double-dose group had a significantly lower ($P < 0.01$) level of Fpg-sensitive sites compared to the control group after the intervention [164]. The level of Fpg-sensitive sites after administration was significantly negatively correlated to the change in serum γ-tocopherol concentration ($r = -0.30$, $n = 60$, $P = 0.018$) [164].

8.3.1 Season

It can be argued that it is more relevant to compare the double-dose group with the control group after treatment since it has been observed that levels of DNA damage vary with season [170-173]. In order to account for seasonal variations, a parallel study design is preferred to a sequential study design [174]. A parallel design was used in the randomised placebo-controlled intervention study in papers IV and V, which was performed between April and June in year 2000. The subjects had been instructed to keep their food habits and level of physical activity stable during the study. However, it is likely that there was a change in exposure to sunlight during that period (spring-early summer), and an association between exposure to sunlight and DNA damage have been observed in several studies [171-173]. It can be speculated that the small (non-significant) increase of Fpg-sensitive sites in the placebo-group was caused by exposure to sunlight, and that the significantly (11.7%) lower level of Fpg-sensitive sites in the double-dose group was due to a protective effect of the antioxidant supplement. Although the evidence of a protective effect of the antioxidant supplement can hardly be regarded as strong and the observed correlation between change of serum γ-tocopherol and Fpg-sensitive sites was weak, it supported the hypothesis that γ-tocopherol could be an important protective agent against oxidative damage [164]. The protective property of γ-tocopherol was further studied in a cell study in paper IV (see section 8.3.5)[164].

8.3.2 The lack of correlation between different methods reflecting oxidative stress

There was no correlation between any of the measured biomarkers of oxidative stress in paper IV. Since they all originate from oxidative damage, the complete lack of correlation is somewhat surprising. Discrepancies between different methods to assess DNA lesions or other methods of oxidative stress are however common. Although it cannot be excluded that a part of this is attributed to experimental variability, it is important to note that different agents cause different types and patterns of oxidative damage. In a summary of dietary intervention studies where comet assay have been

applied, Hoelzl *et al.* found that about half of the published studies found a protective effect against induced DNA damage and/or a decreased level of oxidatively damaged DNA [175]. However, the overlap of positive results in 32 studies where DNA breaks, oxidatively damaged DNA (Fpg-sensitive sites, EndoIII-sensitive sites or both) and protection against induced DNA damage had been measured with the comet assay was poor, i.e. only 3 (10%) of the studies found a positive result in all parameters [175].

Since 8-oxoguanine is Fpg's main substrate, it might seem particularly strange that there is no correlation between 8-oxodG measured by HPLC-EC and Fpg-sensitive sites measured by the comet assay in paper IV. A similar lack of accordance between the level of Fpg-sensitive sites and 8-oxodG/dG was observed by ESCODD when comparing the two methods [67]. There are several differences between the techniques that might explain these discrepancies. First, the HPLC-EC method is susceptible to artefact oxidation. The workup procedure in the HPLC techniques involves a relatively rough protocol, including about 8-10 centrifugation steps as well as purification and hydrolysis of DNA [81]. Artefact oxidation is less critical for the comet assay since it requires only one or a few centrifugation steps and DNA is not purified [81, 176]. ESCODD reported that the variation in background levels were higher when assessed by HPLC-EC compared to when assessed by comet assay (discussed in section 4.1). Secondly, although 8-oxoguanine is generally considered to be Fpg's main substrate *in vivo*, the enzyme is not very specific, and in addition to 8-oxoguanine, it recognises a range of other lesions (see section 3.2.4). Actually, Fpg has even been suggested to recognise lesions resulting from alkylation damage [44]. Due to its higher sensitivity, it can be argued that the comet assay is the preferred method when investigating background levels of damage in humans. It is possible that oxidatively damaged DNA is underestimated when assessed by the comet assay. Lesions in close proximity can appear as only one strand break and some positions of the lesions within the DNA might be less accessible to the enzyme [65, 177, 178]. However, findings by Gedik *et al.* indicate that comet assay and HPLC-EC detects oxidative DNA lesions with similar efficiencies, which contradict an underestimation of damage with the comet assay [68].

8.3.3 Duration, dose and subjects

It cannot be excluded that either the duration and/or the dose of antioxidants were insufficient in the intervention study in paper IV. It is possible that the decrease of Fpg-sensitive sites after antioxidant supplementation would have been significant if more subjects had been included in the study. Møller & Loft argue that the most likely effect of an antioxidant intervention study in healthy subjects would be less than a 10% difference in DNA lesions, and therefore, the number of subjects should be in the hundreds in order to get an appropriate power [147]. However, 89% of the intervention

trials with antioxidants applying comet assay, published between 1996 and 2008, included ≤ 60 subjects [175]. Similarly, the duration of the intervention study in paper IV is relatively long. Hoelzl *et al.* reported in 2009 that about 70% of the published antioxidant intervention studies applying comet assay lasted less than 4 weeks [175].

8.3.4 Comparisons with other studies

The absence of an effect on oxidative stress after antioxidant intervention observed in paper IV is supported by several intervention studies with comparable designs that all report null findings.

Multivitamin supplements

In a parallel placebo-controlled intervention study with antioxidant supplement roughly corresponding to the content found in 600 g of fruit and vegetables, Møller *et al.* found no significant effect on Fpg-sensitive sites or EndoIII-sensitive sites nor on protection against H_2O_2-induced DNA breaks in healthy men and women following a 24 day-intervention [150]. Also, we have performed another placebo-controlled randomised intervention study similar to the study in paper IV, in which subjects with type 2 diabetes was supplemented with the same antioxidant supplement as in paper IV, albeit in a doubled dose, and for a doubled time (12 weeks) [155]. Antioxidant supplementation did not affect the level of any of the six measured biomarkers of oxidative stress or inflammation, in spite of highly significantly elevated levels of serum/plasma levels of six out of seven measured antioxidants [155]. As in the intervention study in paper IV, there was no significantly elevated level of α-tocopherol [155].

Diets rich in antioxidants

In a randomised placebo-controlled cross-over study, male smokers were supplemented with a vegetable and chicken burger and a fruit juice or a chicken burger and a placebo drink for 3 weeks [156]. In spite of highly significantly elevated serum levels of vitamin C and several carotenoids, the antioxidant treatment did not affect the level of EndoIII-sensitive sites or the level of lipid peroxidation (MDA and 8-epi-prostaglandin $F_{2\alpha}$) nor did it protect against H_2O_2-induced DNA breaks [156]. In a controlled intervention study where healthy men and women consumed 600 g of fruit and vegetables daily for 24 days, the intervention did not significantly affect the levels of Fpg-sensitive sites or EndoIII-sensitive sites nor did it protect against H_2O_2-induced DNA breaks [150]. In a recent large controlled randomised parallel intervention study where healthy male smokers consumed a diet rich in antioxidants, i.e. a variety of different juices, berries, fruits, vegetables and nuts, or a controlled normal diet, daily during eight weeks, the intervention did not affect the levels of EndoIII- or Fpg-sensitive sites, nor did it protect against H_2O_2-induced DNA breaks [163].

8.3.5 Vitamin E

Vitamin E is a group of eight structurally related fat-soluble vitamins, four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ), acting as antioxidants by preventing the propagation of free radical reactions by donating hydrogen from their phenolic group to stabilise the radicals, and thereby break the chain of events leading to oxidative damage. The tocopherols protect the structure and function of human cell membranes [179]. Vitamin E supplements often contain only α-tocopherol as for example in the SELECT, ATBC, SU.VI.MAX and the Linxian general population trials described in section 7.1. The reason for this is that the bioactivity and bioavailability of α-tocopherol is higher than that of γ-tocopherol and the other vitamin E compounds, and that α-tocopherol is more abundant in plasma than the other vitamin E compounds. However, the protective effect of oral supplementation of α-tocopherol has been questioned since supplementation with α-tocopherol has been demonstrated to decrease the level of the other vitamin E compounds, and several studies indicate that γ-tocopherol, alone or in combination with α-tocopherol, was important in the protection against oxidative stress [180-182]. Also, several studies indicate that γ-tocopherol has properties not shared by α-tocopherol [183-186]. Chronic inflammation-related diseases are associated with an increased reactive nitrogen species (RNS) formation [187], which γ-tocopherol is particularly efficient in removing [183, 184].

Based on the interesting relationship observed between γ-tocopherol and Fpg-sensitive sites in the intervention study in paper IV, and on the questioned effect of supplementation with α-tocopherol [188], an *in vitro* cell study was performed to investigate the potential of γ-tocopherol and α-tocopherol to protect against induced DNA damage (paper V) [164]. A549 cells that had been pre-incubated with α-tocopherol or γ-tocopherol for 23 h, were exposed to H_2O_2 or peroxynitrite ($ONOO^-$). After 1 h of incubation, the cells were analysed with the comet assay. γ-tocopherol protected against both H_2O_2- and $ONOO^-$-induced oxidative DNA damage in a dose-dependent manner [164]. As little as 5 μM of γ-tocopherol significantly protected DNA against $ONOO^-$-induced oxidative DNA damage [164]. This is close to the levels found in plasma (3 μM) [182]. γ-tocopherol, as opposed to α-tocopherol, has an un-substituted 5'-carbon position on the chromanol ring available for reaction. It is possible that a part of the protective effect of γ-tocopherol against peroxynitrite in the *in vitro* cell study is explained by the formation of the stable adduct 5-nitro-γ-tocopherol [183-185]. The lack of protection by α-tocopherol was surprising. It is a weakness that *RRR*-α-tocopherol was not used instead of *all-rac*-α-tocopherol, which could be speculated to have a lower biopotency than the naturally occurring *RRR*-α-tocopherol. However, this is unlikely to explain the

complete lack of protective effect by α-tocopherol. When designing intervention studies, α-tocopherol's potential to decrease the serum levels of the other potentially important vitamin E compounds should be considered.

9 CONCLUDING DISCUSSION

9.1 COMET ASSAY

Comet assay is a useful tool for biomonitoring studies and the advantages of it include; **a)** that a small cell sample is required, **b)** the possibility to measure damage in practically any cell type, **c)** the ability to measure heterogeneity in response within a cell population, and **d)** the relatively fast and economical procedure. When evaluating information obtained by comet assay the methodological procedures must be taken into account. Dušinská & Collins points out that the value of comet assay depends on how well it is learned and performed [174]. The ECVAG trials indicate that the participants can detect dose-responses of both DNA breaks and Fpg-sensitive sites in coded cells, but that there is a large variation in the reported values [69, 70]. Perhaps the largest surprise in the ECVAG studies was the large inter-laboratory variation in assessment of pre-made slides, indicating that a large fraction of the observed variation is attributed to differences in scoring [69]. Although ECVAG study 2 (paper II) was not designed to investigate differences in protocols, several important parameters in the protocol were shown to significantly affect DNA migration [70]. In paper III we verified that several protocol steps clearly affected the level of DNA migration in the comet assay, including **a)** agarose gel density, **b)** incubation time with Fpg, **c)** duration of alkaline treatment, and **d)** duration of electrophoresis, as well as the strength of the electric field applied [72]. By adjusting the primary endpoints to standard curves, i.e. reference samples analysed with the same protocol, both the inter-laboratory differences [69, 70] and differences between protocols [72] could be decreased substantially, although not completely removed. A possible explanation to this is that the relative sensitivity towards alkaline treatment differs between samples that contain different amounts of ALS [72].

With its multiple applications, comet assay is an important tool contributing to a detailed understanding of genotoxicity and individual susceptibility towards different exposures. However, there is certainly a need for further validation studies in order to reduce both assay variability and inter-laboratory variability. Although useful, comet assay will not reach its full potential until it has been fully validated.

9.2 ANTIOXIDANTS

It is clear that many intervention studies with antioxidants with good design report null findings. It is possible that healthy subjects cannot benefit from an intervention with antioxidants, as indicated by the large clinical placebo-controlled intervention studies in section 7.1. The scientific community has definitely started to question the antioxidant hypothesis, and Collins stated in 2009 that, at the very least, the antioxidant hypothesis

is an over-simplification [81]. Halliwell recently discussed that antioxidant supplements rarely affect the overall level of oxidative damage and subsequently, it is not surprising that human intervention studies with disease or mortality as endpoints have failed to demonstrate the protective effect of antioxidants [2]. Halliwell argues that the endogenously synthesised antioxidants, e.g. superoxide dismutase, peroxyredoxins and reduced glutathione, are far more important for cellular redox balance than the dietary antioxidants [2]. A balanced situation allows some reactive species to perform important tasks, with as little oxidative damage as possible [2].

It should be emphasised that an increased level of antioxidants following antioxidant supplementation, as observed by us in paper IV and in many other studies, is not a proof of a beneficial effect. If the supplemented antioxidants fail to modify the levels of oxidative damage to DNA and lipids, and large randomised placebo-controlled antioxidant supplementation studies fails to prevent disease (in healthy subjects), maybe it is time to start to re-evaluate the design of intervention studies. Lately, an interesting new approach is to try to estimate individual susceptibility in addition to estimating biomarkers of exposure. Measurement of repair capacity and polymorphisms in important enzymes provide new pieces to the puzzle and contribute to the understanding of the cellular redox balance.

In an important review article about dietary antioxidants and oxidatively damaged DNA (in white blood cells and urine), Møller & Loft assigned intervention studies scores based on the quality of the design, ranging from 0 (weak design) to 3 (strong design) [147]. 72 intervention studies had applied repeated dosing, but only 36 of these had a sufficiently good design to be eligible for a restricted analysis [147]. Interestingly, when investigating all 72 intervention studies, and when investigating only the 36 studies with a solid design and realistic baseline values, the same significant pattern was observed; the studies with best study design were significantly more likely to produce null results [147]. Still, about half of the studies with solid design reported preventive effects of antioxidants supplementation, indicating that long-term antioxidant supplementation indeed reduces the levels of oxidative DNA lesions [147]. However, the authors emphasise that the beneficial effect is weak [147].

In a collaborative project performed by the World Cancer Research Fund and the American Institute for Cancer Research, an expert panel of some of the world's leading scientists has performed a large systematic review of the literature aiming to provide recommendations to reduce the risk for cancer [119]. The investigators concluded that dietary supplements are not recommended for cancer prevention and that it is preferred to achieve nutritional adequacy through the usual diet [119]. However, dietary supplements may at times may be beneficial for specific groups, due to dietary

inadequacy or illness [119]. Although there are obviously differences between antioxidant supplements, it is the author of this thesis' opinion that it is much preferred to consume antioxidants via a balanced diet than by antioxidant supplements. Particular circumstances such as medication or deficiency can, however, justify the need for supplements.

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11 REFERENCES

1. Djordjevic VB. Free radicals in cell biology. *Int Rev Cytol,* (2004) **237**, 57-89.

2. Halliwell B. Free radicals and antioxidants - quo vadis? *Trends Pharmacol Sci,* (2011) **32**, 125-130.

3. Halliwell B, Gutteridge J. *Free Radicals in Biology and Medicine. 3rd ed.* Oxford, Oxford University Press, 1999.

4. Sies H. *Oxidative stress II. Oxidants and Antioxidants.* London, Academic Press, 1991.

5. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med,* (2001) **30**, 1191-1212.

6. Kasai H, Nishimura S. Hydroxylation of Deoxyguanosine at the C-8 Position by Ascorbic-Acid and Other Reducing Agents. *Nucleic Acids Res,* (1984) **12**, 2137-2145.

7. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J,* (2003) **17**, 1195-1214.

8. Møller P. The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. *Basic Clin Pharmacol Toxicol,* (2006) **98**, 336-345.

9. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol,* (2004) **26**, 249-261.

10. Dušinská M, Collins A. Detection of oxidised purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay. *ATLA,* (1996) **24**, 405-411.

11. Collins AR, Duthie SJ, Dobson VL. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis,* (1993) **14**, 1733-1735.

12. Smith CC, O'Donovan MR, Martin EA. hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis,* (2006) **21**, 185-190.

13. Collins AR, Dušinská M, Horska A. Detection of alkylation damage in human lymphocyte DNA with the comet assay. *Acta Biochim Pol,* (2001) **48**, 611-614.

14. Collins AR, Mitchell DL, Zunino A, deWit J, Busch D. UV-sensitive rodent mutant cell lines of complementation groups 6 and 8 differ phenotypically from their human counterparts. *Environ Mol Mutagen,* (1997) **29**, 152-160.

15. Gedik CM, Ewen SW, Collins AR. Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells. *Int J Radiat Biol,* (1992) **62**, 313-320.

16. Duthie SJ, McMillan P. Uracil misincorporation in human DNA detected using single cell gel electrophoresis. *Carcinogenesis,* (1997) **18**, 1709-1714.

17. Collins AR, Dušinská M, Horvathova E, Munro E, Savio M, Stetina R. Inter-individual differences in repair of DNA base oxidation, measured in vitro with the comet assay. *Mutagenesis,* (2001) **16**, 297-301.

18. Langie SA, Knaapen AM, Brauers KJ, van Berlo D, van Schooten FJ, Godschalk RW. Development and validation of a modified comet assay to phenotypically assess nucleotide excision repair. *Mutagenesis,* (2006) **21**, 153-158.

19. Gaivao I, Piasek A, Brevik A, Shaposhnikov S, Collins AR. Comet assay-based methods for measuring DNA repair in vitro; estimates of inter- and intra-individual variation. *Cell Biol Toxicol,* (2009) **25**, 45-52.

20. Herrera M, Dominguez G, Garcia JM, Pena C, Jimenez C, Silva J, Garcia V, Gomez I, et al. Differences in repair of DNA cross-links between lymphocytes and epithelial tumor cells from colon cancer patients measured in vitro with the comet assay. *Clin Cancer Res,* (2009) **15**, 5466-5472.
21. Spanswick VJ, Hartley JM, Hartley JA. Measurement of DNA interstrand crosslinking in individual cells using the Single Cell Gel Electrophoresis (Comet) assay. *Methods Mol Biol,* (2010) **613**, 267-282.
22. Shaposhnikov S, Thomsen PD, Collins AR. Combining fluorescent in situ hybridization with the comet assay for targeted examination of DNA damage and repair. *Methods Mol Biol,* (2011) **682**, 115-132.
23. Tice R. The single cell gel/comet assay: A microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In: Phillips DH, Venitt S (Eds). *Environmental Mutagenesis*. Oxford, Bios Scientific Publishers, 1995, Ch. 6, pp. 315–339.
24. Kirkland D, Speit G. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens - III. Appropriate follow-up testing in vivo. *Mutat Res,* (2008) **654**, 114-132.
25. Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database. *Crit Rev Toxicol,* (2000) **30**, 629-799.
26. Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith CC, Stetina R. The comet assay: topical issues. *Mutagenesis,* (2008) **23**, 143-151.
27. Albertini RJ, Anderson D, Douglas GR, Hagmar L, Hemminki K, Merlo F, Natarajan AT, Norppa H, et al. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutat Res,* (2000) **463**, 111-172.
28. Collins AR. The use of bacterial repair endonucleases in the comet assay. *Methods Mol Biol,* (2011) **691**, 137-147.
29. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, et al. Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop. *Mutagenesis,* (2003) **18**, 45-51.
30. Rojas E, Lopez MC, Valverde M. Single cell gel electrophoresis assay: methodology and applications. *J Chromatogr B Biomed Sci Appl,* (1999) **722**, 225-254.
31. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen,* (2000) **35,** 206-221.
32. Östling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun,* (1984) **123**, 291-298.
33. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res,* (1988) **175**, 184-191.
34. Olive PL. Cell proliferation as a requirement for development of the contact effect in Chinese hamster V79 spheroids. *Radiat Res,* (1989) **117**, 79-92.
35. Olive PL, Banath JP, Durand RE. Detection of Etoposide Resistance by Measuring DNA Damage in Individual Chinese Hamster-Cells. *J Natl Cancer Inst,* (1990) **82**, 779-783.

36. Al-Salmani K, Abbas HH, Schulpen S, Karbaschi M, Abdalla I, Bowman KJ, So KK, Evans MD, et al. Simplified method for the collection, storage, and comet assay analysis of DNA damage in whole blood. *Free Radic Biol Med,* (2011) **51**, 719-725.
37. Shaposhnikov S, Azqueta A, Henriksson S, Meier S, Gaivao I, Huskisson NH, Smart A, Brunborg G, et al. Twelve-gel slide format optimised for comet assay and fluorescent in situ hybridisation. *Toxicol Lett,* (2010) **195**, 31-34.
38. Singh NP. Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat Res,* (2000) **455**, 111-127.
39. Friedman JI, Stivers JT. Detection of damaged DNA bases by DNA glycosylase enzymes. *Biochemistry,* (2010) **49**, 4957-4967.
40. Stivers JT, Jiang YL. A mechanistic perspective on the chemistry of DNA repair glycosylases. *Chemical Rev,* (2003) **103**, 2729-2759.
41. Boiteux S, Gajewski E, Laval J, Dizdaroglu M. Substrate specificity of the Escherichia coli Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionizing radiation or photosensitization. *Biochemistry,* (1992) **31**, 106-110.
42. Tchou J, Bodepudi V, Shibutani S, Antoshechkin I, Miller J, Grollman AP, Johnson F. Substrate specificity of Fpg protein. Recognition and cleavage of oxidatively damaged DNA. *J Biol Chem,* (1994) **269**, 15318-15324.
43. David SS, Williams SD. Chemistry of Glycosylases and Endonucleases Involved in Base-Excision Repair. *Chem Rev,* (1998) **98**, 1221-1262.
44. Speit G, Schutz P, Bonzheim I, Trenz K, Hoffmann H. Sensitivity of the FPG protein towards alkylation damage in the comet assay. *Toxicol Lett,* (2004) **146**, 151-158.
45. David SS, O'Shea VL, Kundu S. Base-excision repair of oxidative DNA damage. *Nature,* (2007) **447**, 941-950.
46. Hamm ML, Gill TJ, Nicolson SC, Summers MR. Substrate specificity of Fpg (MutM) and hOGG1, two repair glycosylases. *J Am Chem Soc,* (2007) **129**, 7724-7725.
47. Hatahet Z, Kow YW, Purmal AA, Cunningham RP, Wallace SS. New substrates for old enzymes. 5-Hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine are substrates for Escherichia coli endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2'-deoxyuridine is a substrate for uracil DNA N-glycosylase. *J Biol Chem,* (1994) **269**, 18814-18820.
48. Boiteux S, Radicella JP. Base excision repair of 8-hydroxyguanine protects DNA from endogenous oxidative stress. *Biochimie,* (1999) **81**, 59-67.
49. Kuznetsov NA, Koval VV, Fedorova OS. Mechanism of recognition and repair of damaged DNA by human 8-oxoguanine DNA glycosylase hOGG1. *Biochemistry (Mosc),* (2011) **76**, 118-130.
50. O'Brien PJ, Ellenberger T. The Escherichia coli 3-methyladenine DNA glycosylase AlkA has a remarkably versatile active site. *J Biol Chem,* (2004) **279**, 26876-26884.
51. Zharkov DO, Mechetin GV, Nevinsky GA. Uracil-DNA glycosylase: Structural, thermodynamic and kinetic aspects of lesion search and recognition. *Mutat Res,* (2010) **685**, 11-20.
52. Collins AR, Azqueta A. DNA repair as a biomarker in human biomonitoring studies; further applications of the comet assay. *Mutat Res,* (2011) doi:10.1016/j.mrfmmm.2011.03.005.

53. Shaposhnikov SA, Salenko VB, Brunborg G, Nygren J, Collins AR. Single-cell gel electrophoresis (the comet assay): Loops or fragments? *Electrophoresis,* (2008) **29**, 3005-3012.
54. Azqueta A, Meier S, Priestley C, Gutzkow KB, Brunborg G, Sallette J, Soussaline F, Collins A. The influence of scoring method on variability in results obtained with the comet assay. *Mutagenesis,* (2011) **26**, 393-399.
55. Horvathova E, Dušinská M, Shaposhnikov S, Collins AR. DNA damage and repair measured in different genomic regions using the comet assay with fluorescent in situ hybridization. *Mutagenesis,* (2004) **19**, 269-276.
56. Santos SJ, Singh NP, Natarajan AT. Fluorescence in situ hybridization with comets. *Exp Cell Res,* (1997) **232**, 407-411.
57. Pfuhler S, Wolf HU. Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ Mol Mutagen,* (1996) **27**, 196-201.
58. Dušinská M, Collins AR. The comet assay in human biomonitoring: gene-environment interactions. *Mutagenesis,* (2008) **23**, 191-205.
59. Rothman N, Stewart WF, Rabkin CS, Schulte PA. The development, validation, and application of biomarkers for early biologic effects. In: Mendelsohn ML, Peeters JP, Normandy MJ (Eds). *Biomarkers and Occupational Health: Progress and Perspectives.* Washington DC, Joseph Henry Press, 1995, pp. 109–115.
60. Collins A, Cadet J, Epe B, Gedik C. Problems in the measurement of 8-oxoguanine in human DNA. Report of a workshop, DNA Oxidation, held in Aberdeen, UK, 19-21 January, 1997. *Carcinogenesis,* (1997) **18**, 1833-1836.
61. Lunec J. ESCODD: European Standards Committee on Oxidative DNA damage. *Free Radic Res,* (1998) **29**, 601-608.
62. ESCODD. Comparison of different methods of measuring 8-oxoguanine as a marker of oxidative DNA damage. ESCODD (European Standards Committee on Oxidative DNA Damage). *Free Radic Res,* (2000) **32**, 333-341.
63. Riis B. Comparison of results from different laboratories in measuring 8-oxo-2'-deoxyguanosine in synthetic oligonucleotides. *Free Radic Res,* (2002) **36**, 649-659.
64. ESCODD. Inter-laboratory validation of procedures for measuring 8-oxo-7,8-dihydroguanine/8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA. *Free Radic Res,* (2002) **36**, 239-245.
65. ESCODD. Comparative analysis of baseline 8-oxo-7,8-dihydroguanine in mammalian cell DNA, by different methods in different laboratories: an approach to consensus. *Carcinogenesis,* (2002) **23**, 2129-2133.
66. ESCODD. Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. *Free Radic Biol Med,* (2003) **34**, 1089-1099.
67. Gedik CM, Collins A; ESCODD. Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study. *FASEB J,* (2005) **19**, 82-84.
68. Gedik CM, Boyle SP, Wood SG, Vaughan NJ, Collins AR. Oxidative stress in humans: validation of biomarkers of DNA damage. *Carcinogenesis,* (2002) **23**, 1441-1446.
69. Forchhammer L, Johansson C, Loft S, Möller L, Godschalk RW, Langie SA, Jones GD, Kwok RW, et al. Variation in the measurement of DNA damage by comet assay measured by the ECVAG inter-laboratory validation trial. *Mutagenesis,* (2010) **25**, 113-123.
70. Johansson C, Møller P, Forchhammer L, Loft S, Godschalk RW, Langie SA, Lumeij S, Jones GD, et al. An ECVAG trial on assessment of oxidative damage to DNA measured by the comet assay. *Mutagenesis,* (2010) **25**, 125-132.

71. Zainol M, Stoute J, Almeida GM, Rapp A, Bowman KJ, Jones GDD, ECVAG. Introducing a true internal standard for the Comet assay to minimize intra- and inter-experiment variability in measures of DNA damage and repair. *Nucleic Acids Res,* (2009) **37**, e150.
72. Ersson C, Möller L. The effects on DNA migration of altering parameters in the comet assay protocol such as agarose density, electrophoresis conditions, and durations of the enzyme or the alkaline treatments. *Mutagenesis,* (2011) doi: 10.1093/mutage/ger034.
73. Speit G, Trenz K, Schutz P, Rothfuss A, Merk O. The influence of temperature during alkaline treatment and electrophoresis on results obtained with the comet assay. *Toxicol Lett,* (1999) **110**, 73-78.
74. Azqueta A, Gutzkow KB, Brunborg G, Collins AR. Towards a more reliable comet assay: Optimising agarose concentration, unwinding time and electrophoresis conditions. *Mutat Res,* (2011) **724,** 41-45.
75. Bauch T, Böcker W, Mallek U, Müller WU, Streffer C. Optimization and standardization of the "comet assay" for analyzing the repair of DNA damage in cells. *Strahlenther Onkol,* (1999) **175**, 333-340.
76. Available from NewGeneris homepage [online]: http://www.newgeneris.org/ [16 June 2011].
77. Azqueta A, Shaposhnikov S, Collins AR. DNA oxidation: Investigating its key role in environmental mutagenesis with the comet assay. *Mutat Res,* (2009) **674**, 101-108.
78. Kohn KW. Principles and practice of DNA filter elution. *Pharmacol Ther,* (1991) **49**, 55-77.
79. Forchhammer L, Bräuner EV, Folkmann JK, Danielsen PH, Nielsen C, Jensen A, Loft S, Friis G, Møller P. Variation in assessment of oxidatively damaged DNA in mononuclear blood cells by the comet assay with visual scoring. *Mutagenesis,* (2008) **23**, 223-231.
80. Vijayalaxmi, Tice RR, Strauss GHS. Assessment of radiation-induced DNA damage in human blood lymphocytes using the single-cell gel electrophoresis technique. *Mutat Res,* (1992) **271**, 243-252.
81. Collins AR. Investigating oxidative DNA damage and its repair using the comet assay. *Mutat Res,* (2009) **681**, 24-32.
82. Will O, Gocke E, Eckert I, Schulz I, Pflaum M, Mahler HC, Epe B. Oxidative DNA damage and mutations induced by a polar photosensitizer, Ro19-8022. *Mutat Res,* (1999) **435**, 89-101.
83. Speit G, Hartmann A. The comet assay: a sensitive genotoxicity test for the detection of DNA damage and repair. *Methods Mol Biol,* (2006) **314**, 275-286.
84. Collins AR, Dušinská M. Oxidation of cellular DNA measured with the comet assay. *Methods Mol Biol,* (2002) **186**, 147-159.
85. Available from COMICS homepage [online]: http://comics.vitamib.com/electrophoresis-physics [15 June 2011].
86. Collins A, Dušinská M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, et al. Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ Mol Mutagen,* (1997) **30**, 139-146.
87. Garcia O, Mandina T, Lamadrid AI, Diaz A, Remigio A, Gonzalez Y, Piloto J, Gonzalez JE, et al. Sensitivity and variability of visual scoring in the comet assay. Results of an inter-laboratory scoring exercise with the use of silver staining. *Mutat Res,* (2004) **556**, 25-34.
88. Møller P, Friis G, Christensen PH, Risom L, Plesner G, Kjaersgaard J, Vinzents P, Loft S, et al. Intra-laboratory comet assay sample scoring exercise for

determination of formamidopyrimidine DNA glycosylase sites in human mononuclear blood cell DNA. *Free Radic Res,* (2004) **38**, 1207-1214.
89. Tice RR, Strauss GH. The single cell gel electrophoresis/comet assay: a potential tool for detecting radiation-induced DNA damage in humans. *Stem Cells,* (1995) **13,** Suppl 1, 207-214.
90. Hellman B, Vaghef H, Boström B. The concepts of tail moment and tail inertia in the single cell gel electrophoresis assay. *Mutat Res,* (1995) **336**, 123-131.
91. Olive PL, Banath JP. Induction and rejoining of radiation-induced DNA single-strand breaks: "tail moment" as a function of position in the cell cycle. *Mutat Res,* (1993) **294**, 275-283.
92. Karlsson HL, Nygren J, Möller L. Genotoxicity of airborne particulate matter: the role of cell-particle interaction and of substances with adduct-forming and oxidizing capacity. *Mutat Res,* (2004) **565**, 1-10.
93. Olive PL. The comet assay. An overview of techniques. *Methods Mol Biol,* (2002) **203**, 179-194.
94. Collins AR, Dušinská M, Gedik CM, Stetina R. Oxidative damage to DNA: do we have a reliable biomarker? *Environ Health Perspect,* (1996) **104** Suppl 3, 465-469.
95. Kohn KW, Erickson LC, Ewig RA, Friedman CA. Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry,* (1976) **15**, 4629-4637.
96. Ahnström G, Erixon K. In: Friedberg EC, Hanawalt PC (Eds). *DNA Repair: A Laboratory Manual of Research Procedures.* New York, Marcel Dekker, 1981, pp. 403−418
97. Malyapa RS, Bi C, Ahern EW, Roti Roti JL. Detection of DNA damage by the alkaline comet assay after exposure to low-dose gamma radiation. *Radiat Res,* (1998) **149**, 396-400.
98. Pflaum M, Will O, Epe B. Determination of steady-state levels of oxidative DNA base modifications in mammalian cells by means of repair endonucleases. *Carcinogenesis,* (1997) **18**, 2225-2231.
99. Ersson C, Thorman R, Rodhe Y, Möller L, Hylander B. DNA damage in salivary gland tissue in patients with chronic kidney disease, measured by the comet assay. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod,* (2011) **112**, 209-215.
100. Karlsson HL. The comet assay in nanotoxicology research. *Anal Bioanal Chem,* (2010) **398**, 651-666.
101. Stone V, Johnston H, Schins RPF. Development of in vitro systems for nanotoxicology: methodological considerations. *Crit Rev Toxicol,* (2009) **39**, 613-626.
102. Asmuss M, Mullenders LHF, Eker A, Hartwig A. Differential effects of toxic metal compounds on the activities of Fpg and XPA, two zinc finger proteins involved in DNA repair. *Carcinogenesis,* (2000) **21**, 2097-2104.
103. Hirano T. Repair system of 7,8-dihydro-8-oxoguanine as a defense line against carcinogenesis. *J Radiat Res (Tokyo),* (2008) **49**, 329-340.
104. Halliwell B. Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. *Nutr Rev,* (1999) **57**, 104-113.
105. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. *Mutat Res,* (2004) **567**, 447-474.
106. Bonassi S, Norppa H, Ceppi M, Strömberg U, Vermeulen R, Znaor A, Cebulska-Wasilewska A, Fabianova E, et al. Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries. *Carcinogenesis,* (2008) **29**, 1178-1183.

107. Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, et al. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis,* (2007) **28**, 625-631.

108. Hagmar L, Bonassi S, Strömberg U, Brøgger A, Knudsen LE, Norppa H, Reuterwall C. Chromosomal aberrations in lymphocytes predict human cancer: A report from the European study group on cytogenetic biomarkers and health (ESCH). *Cancer Res,* (1998) **58**, 4117-4121.

109. Neeley WL, Essigmann JM. Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. *Chem Res Toxicol,* (2006) **19**, 491-505.

110. Kasai H. Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res,* (1997) **387**, 147-163.

111. Pouget JP, Douki T, Richard MJ, Cadet J. DNA damage induced in cells by gamma and UVA radiation as measured by HPLC/GC-MS and HPLC-EC and Comet assay. *Chem Res Toxicol,* (2000) **13**, 541-549.

112. Hsu GW, Ober M, Carell T, Beese LS. Error-prone replication of oxidatively damaged DNA by a high-fidelity DNA polymerase. *Nature,* (2004) **431**, 217-221.

113. Moriya M, Ou C, Bodepudi V, Johnson F, Takeshita M, Grollman AP. Site-specific mutagenesis using a gapped duplex vector: a study of translesion synthesis past 8-oxodeoxyguanosine in E. coli. *Mutat Res,* (1991) **254**, 281-288.

114. Kirouac KN, Ling H. Unique active site promotes error-free replication opposite an 8-oxo-guanine lesion by human DNA polymerase iota. *Proc Natl Acad Sci U S A,* (2011) **108**, 3210-3215.

115. Reardon JT, Bessho T, Kung HC, Bolton PH, Sancar A. In vitro repair of oxidative DNA damage by human nucleotide excision repair system: Possible explanation for neurodegeneration in Xeroderma pigmentosum patients. *Proc Natl Acad Sci U S A,* (1997) **94**, 9463-9468.

116. Hazra TK, Das A, Das S, Choudhury S, Kow YW, Roy R. Oxidative DNA damage repair in mammalian cells: A new perspective. *DNA Repair (Amst),* (2007) **6**, 470-480.

117. Paz-Elizur T, Sevilya Z, Leitner-Dagan Y, Elinger D, Roisman LC, Livneh Z. DNA repair of oxidative DNA damage in human carcinogenesis: Potential application for cancer risk assessment and prevention. *Cancer Lett,* (2008) **266**, 60-72.

118. Azqueta A, Lorenzo Y, Collins AR. In vitro comet assay for DNA repair: a warning concerning application to cultured cells. *Mutagenesis,* (2009) **24**, 379-381.

119. World Cancer Research Fund/American Institute for Cancer Research. *Food, Nutrition, and Physical Activity, and the Prevention of Cancer: A Global Perspective.* Washington DC, AICR, 2007.

120. Ness AR, Powles JW. Fruit and vegetables, and cardiovascular disease: a review. *Int J Epidemiol,* (1997) **26**, 1-13.

121. Bailey RL, Gahche JJ, Lentino CV, Dwyer JT, Engel JS, Thomas PR, Betz JM, Sempos CT, Picciano MF. Dietary supplement use in the United States, 2003-2006. *J Nutr,* (2011) **141**, 261-266.

122. Gahche J, Bailey R, Burt V, Hughes J, Yetley E, Dwyer J, Picciano MF, McDowell M, et al. Dietary supplement use among U.S. adults has increased since NHANES III (1988-1994). *NCHS Data Brief,* (2011), 1-8.

123. National Institutes of Health State-of-the-Science Conference statement: multivitamin/mineral supplements and chronic disease prevention. *Ann Intern Med.* (2006) **145**, 364-371.
124. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA,* (2007) **297**, 842-857.
125. Halliwell B. The antioxidant paradox. *Lancet,* (2000) **355**, 1179-1180.
126. Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, Yang CS, Zheng SF, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *J Natl Cancer Inst,* (1993) **85**, 1483-1492.
127. Blot WJ, Li JY, Taylor PR, Guo WD, Dawsey SM, Li B. The Linxian trials: Mortality rates by vitamin-mineral intervention group. *Am J Clin Nutr,* (1995) **62**, S1424-S1426.
128. Qiao YL, Dawsey SM, Kamangar F, Fan JH, Abnet CC, Sun XD, Johnson LL, Gail MH, et al. Total and cancer mortality after supplementation with vitamins and minerals: Follow-up of the Linxian General Population Nutrition Intervention Trial. *J Natl Cancer Inst,* (2009) **101**, 507-518.
129. Albanes D, Heinonen OP, Huttunen JK, Taylor PR, Virtamo J, Edwards BK, Haapakoski J, Rautalahti M, et al. Effects of alpha-tocopherol and beta-carotene supplements on cancer incidence in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study. *Am J Clin Nutr,* (1995) **62**, S1427-S1430.
130. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta-carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med,* (1994) **330**, 1029-1035.
131. Virtamo J, Pietinen P, Huttunen JK, Malila N, Virtanen MJ, Albanes D, Taylor PR, Albert P. Incidence of cancer and mortality following alpha-tocopherol and beta-carotene supplementation: A postintervention follow-up. *JAMA,* (2003) **290**, 476-485.
132. Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahti M, Hartman AM, Palmgren J, et al. Alpha-tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. *J Natl Cancer Inst,* (1996) **88**, 1560-1570.
133. Goodman GE, Thornquist MD, Balmes J, Cullen MR, Meyskens FL, Omenn GS, Valanis B, Williams JH. The beta-carotene and retinol efficacy trial: Incidence of lung cancer and cardiovascular disease mortality during 6-year follow-up after stopping beta-carotene and retinol supplements. *J Natl Cancer Inst,* (2004) **96**, 1743-1750.
134. Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, et al. Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *J Natl Cancer Inst,* (1996) **88**, 1550-1559.
135. Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med,* (1996) **334**, 1150-1155.
136. Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, Belanger C, LaMotte F, et al. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med,* (1996) **334**, 1145-1149.

137. Hercberg S, Galan P, Preziosi P, Bertrais S, Mennen L, Malvy D, Roussel AM, Favier A, Briancon S. The SU.VI.MAX Study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch Intern Med,* (2004) **164**, 2335-2342.

138. Meyer F, Galan P, Douville P, Bairati I, Kegle P, Bertrais S, Estaquio C, Hercberg S. Antioxidant vitamin and mineral supplementation and prostate cancer prevention in the SU.VI.MAX trial. *Int J Cancer,* (2005) **116**, 182-186.

139. Wactawski-Wende J, Kotchen JM, Anderson GL, Assaf AR, Brunner RL, O'Sullivan MJ, Margolis KL, Ockene JK, et al. Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N Engl J Med,* (2006) **354**, 684-696.

140. Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, Parnes HL, Minasian LM, et al. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA,* (2009) **301**, 39-51.

141. Cook NR, Stampfer MJ, Ma J, Manson JE, Sacks FM, Buring JE, Hennekens CH. Beta-carotene supplementation for patients with low baseline levels and decreased risks of total and prostate carcinoma. *Cancer,* (1999) **86**, 1783-1792.

142. Tran GD, Sun XD, Abnet CC, Fan JH, Dawsey SM, Dong ZW, Mark SD, Qiao YL, Taylor PR. Prospective study of risk factors for esophageal and gastric cancers in the Linxian General Population Trial cohort in China. *Int J Cancer,* (2005) **113**, 456-463.

143. Zou XN, Taylor PR, Mark SD, Chao A, Wang W, Dawsey SM, Wu YP, Qiao YL, et al. Seasonal variation of food consumption and selected nutrient intake,in Linxian, a high risk area for esophageal cancer in China. *Int J Vitam Nutr Res,* (2002) **72**, 375-382.

144. Greenwald P, Anderson D, Nelson SA, Taylor PR. Clinical trials of vitamin and mineral supplements for cancer prevention. *Am J Clin Nutr,* (2007) **85**, 314S-317S.

145. Neuhouser ML, Patterson RE, Thornquist MD, Omenn GS, King IB, Goodman GE. Fruits and vegetables are associated with lower lung cancer risk only in the placebo arm of the beta-carotene and retinol efficacy trial (CARET). *Cancer Epidemiol Biomarkers Prev,* (2003) **12**, 350-358.

146. Møller P, Loft S. Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. *Am J Clin Nutr,* (2002) **76**, 303-310.

147. Møller P, Loft S. Dietary antioxidants and beneficial effect on oxidatively damaged DNA. *Free Radic Biol Med,* (2006) **41**, 388-415.

148. Collins AR, Olmedilla B, Southon S, Granado F, Duthie SJ. Serum carotenoids and oxidative DNA damage in human lymphocytes. *Carcinogenesis,* (1998) **19**, 2159-2162.

149. Boyle SP, Dobson VL, Duthie SJ, Hinselwood DC, Kyle JA, Collins AR. Bioavailability and efficiency of rutin as an antioxidant: a human supplementation study. *Eur J Clin Nutr,* (2000) **54**, 774-782.

150. Møller P, Vogel U, Pedersen A, Dragsted LO, Sandstrom B, Loft S. No effect of 600 grams fruit and vegetables per day on oxidative DNA damage and repair in healthy nonsmokers. *Cancer Epidemiol Biomarkers Prev,* (2003) **12**, 1016-1022.

151. Guarnieri S, Loft S, Riso P, Porrini M, Risom L, Poulsen HE, Dragsted LO, Møller P. DNA repair phenotype and dietary antioxidant supplementation. *Br J Nutr,* (2008) **99**, 1018-1024.

152. Møller P, Viscovich M, Lykkesfeldt J, Loft S, Jensen A, Poulsen HE. Vitamin C supplementation decreases oxidative DNA damage in mononuclear blood cells of smokers. *Eur J Nutr,* (2004) **43**, 267-274.
153. Duthie SJ, Ma A, Ross MA, Collins AR. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Res,* (1996) **56**, 1291-1295.
154. Rytter E, Johansson C, Vessby B, Sjödin A, Möller L, Åkesson B, Basu S. Biomarkers of oxidative stress in overweight men are not influenced by a combination of antioxidants. *Free Radic Res,* (2010) **44**, 522-528.
155. Rytter E, Vessby B, Åsgård R, Ersson C, Moussavian S, Sjödin A, Abramsson-Zetterberg L, Möller L, Basu S. Supplementation with a combination of antioxidants does not affect glycaemic control, oxidative stress or inflammation in type 2 diabetes subjects. *Free Radic Res,* (2010) **44**, 1445-1453.
156. van den Berg R, van Vliet T, Broekmans WM, Cnubben NH, Vaes WH, Roza L, Haenen GR, Bast A, et al. A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of antioxidant status in male smokers. *J Nutr,* (2001) **131**, 1714-1722.
157. Collins AR, Harrington V, Drew J, Melvin R. Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis,* (2003) **24**, 511-515.
158. Brevik A, Gaivao I, Medin T, Jorgenesen A, Piasek A, Elilasson J, Karlsen A, Blomhoff R, et al. Supplementation of a western diet with golden kiwifruits (Actinidia chinensis var.'Hort 16A':) effects on biomarkers of oxidation damage and antioxidant protection. *Nutr J,* (2011) **10**, 54.
159. Møller P, Loft S, Alfthan G, Freese R. Oxidative DNA damage in circulating mononuclear blood cells after ingestion of blackcurrant juice or anthocyanin-rich drink. *Mutat Res,* (2004) **551**, 119-126.
160. Duthie SJ, Jenkinson AM, Crozier A, Mullen W, Pirie L, Kyle J, Yap LS, Christen P, Duthie GG. The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. *Eur J Nutr,* (2006) **45**, 113-122.
161. Riso P, Martini D, Visioli F, Martinetti A, Porrini M. Effect of broccoli intake on markers related to oxidative stress and cancer risk in healthy smokers and nonsmokers. *Nutr Cancer,* (2009) **61**, 232-237.
162. Riso P, Martini D, Møller P, Loft S, Bonacina G, Moro M, Porrini M. DNA damage and repair activity after broccoli intake in young healthy smokers. *Mutagenesis,* (2010) **25**, 595-602.
163. Brevik A, Karlsen A, Azqueta A, Tirado AE, Blomhoff R, Collins A. Both base excision repair and nucleotide excision repair in humans are influenced by nutritional factors. *Cell Biochem Funct,* (2011) **29**, 36-42.
164. Johansson C, Rytter E, Nygren J, Vessby B, Basu S, Möller L. Down-regulation of oxidative DNA lesions in human mononuclear cells after antioxidant supplementation correlates to increase of gamma-tocopherol. *Int J Vitam Nutr Res,* (2008) **78,** 183-194.
165. Hofer T, Möller L. Optimization of the workup procedure for the analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine with electrochemical detection. *Chem Res Toxicol,* (2002) **15**, 426-432.
166. Basu S. Radioimmunoassay of 8-iso-prostaglandin F2alpha: an index for oxidative injury via free radical catalysed lipid peroxidation. *Prostaglandins Leukot Essent Fatty Acids,* (1998) **58**, 319-325.
167. Öhrvall M, Tengblad S, Ekstrand B, Siegbahn A, Vessby B. Malondialdehyde concentration in plasma is inversely correlated to the proportion of linoleic acid in serum lipoprotein lipids. *Atherosclerosis,* (1994) **108**, 103-110.

168. Hofer T. *Thesis: Method Development for Analysis of 8-oxodG as a Biomarker for Oxidative Stress*. Stockholm, Karolinska Institutet, 2001.
169. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis,* (2005) **15**, 316-328.
170. Dušinská M, Vallova B, Ursinyova M, Hladikova V, Smolkova B, Wsolova L, Raslova K, Collins AR. DNA damage and antioxidants; fluctuations through the year in a central European population group. *Food Chem Toxicol,* (2002) **40**, 1119-1123.
171. Møller P, Knudsen LE, Frentz G, Dybdahl M, Wallin H, Nexo BA. Seasonal variation of DNA damage and repair in patients with non-melanoma skin cancer and referents with and without psoriasis. *Mutat Res,* (1998) **407**, 25-34.
172. Møller P, Wallin H, Holst E, Knudsen LE. Sunlight-induced DNA damage in human mononuclear cells. *FASEB J,* (2002) **16**, 45-53.
173. Tsilimigaki SI, Messini-Nikolaki N, Kanariou M, Piperakis SM. A study on the effects of seasonal solar radiation on exposed populations. *Mutagenesis,* (2003) **18**, 139-143.
174. Dušinská M, Collins AR. DNA Oxidation, Antioxidant Effects, and DNA Repair Measured with the Comet Assay. In: Aldini G, Yeum KJ, Niki E, Russell RM (Eds). *Biomarkers for Antioxidant Defense and Oxidative Damage: Principles and Practical Applications*: Blackwell Publishing Ltd, 2010, pp. 261-282.
175. Hoelzl C, Knasmuller S, Misik M, Collins A, Dušinská M, Nersesyan A. Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: Recent results and trends. *Mutat Res,* (2009) **681**, 68-79.
176. Pouget JP, Ravanat JL, Douki T, Richard MJ, Cadet J. Measurement of DNA base damage in cells exposed to low doses of gamma-radiation: comparison between the HPLC-EC and comet assays. *Int J Radiat Biol,* (1999) **75**, 51-58.
177. Gedik CM, Wood SG, Collins AR. Measuring oxidative damage to DNA; HPLC and the comet assay compared. *Free Radic Res,* (1998) **29**, 609-615.
178. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol,* (2004) **142**, 231-255.
179. Elmadfa I, Wagner KH. Non-nutritive bioactive food constituents of plants: tocopherols (vitamin E). *Int J Vitam Nutr Res,* (2003) **73**, 89-94.
180. Devaraj S, Leonard S, Traber MG, Jialal I. Gamma-tocopherol supplementation alone and in combination with alpha-tocopherol alters biomarkers of oxidative stress and inflammation in subjects with metabolic syndrome. *Free Radic Biol Med,* (2008) **44**, 1203-1208.
181. Helzlsouer KJ, Huang HY, Alberg AJ, Hoffman S, Burke A, Norkus EP, Morris JS, Comstock GW. Association between alpha-tocopherol, gamma-tocopherol, selenium, and subsequent prostate cancer. *J Natl Cancer Inst,* (2000) **92**, 2018-2023.
182. Hensley K, Benaksas EJ, Bolli R, Comp P, Grammas P, Hamdheydari L, Mou S, Pye QN, et al. New perspectives on vitamin E: gamma-tocopherol and carboxyelthylhydroxychroman metabolites in biology and medicine. *Free Radic Biol Med,* (2004) **36**, 1-15.
183. Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, Ames BN. gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alpha-tocopherol: physiological implications. *Proc Natl Acad Sci U S A,* (1997) **94**, 3217-3222.

184. Cooney RV, Franke AA, Harwood PJ, Hatch-Pigott V, Custer LJ, Mordan LJ. Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol. *Proc Natl Acad Sci U S A,* (1993) **90**, 1771-1775.
185. Morton LW, Ward NC, Croft KD, Puddey IB. Evidence for the nitration of gamma-tocopherol in vivo: 5-nitro-gamma-tocopherol is elevated in the plasma of subjects with coronary heart disease. *Biochem J,* (2002) **364**, 625-628.
186. Saldeen T, Li D, Mehta JL. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. *J Am Coll Cardiol,* (1999) **34**, 1208-1215.
187. Ferguson LR. Chronic inflammation and mutagenesis. *Mutat Res,* (2010) **690**, 3-11.
188. Kline K, Lawson KA, Yu W, Sanders BG. Vitamin E and cancer. *Vitam Horm,* (2007) **76**, 435-461.